

Immunity to *Leishmania mexicana* parasite

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Declaration

“I hereby declare that the dissertation submitted for the Degree of Doctor of Philosophy in immunity to *Leishmania mexicana* parasite at Nottingham Trent University, is my own original work and has not previously been submitted to any Institution or University or quoted as indicated and acknowledged by means of a comprehensive list of references”

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Abstract

Background: Cutaneous Leishmaniasis caused by the *Leishmania mexicana* complex is associated with unpleasant or disfiguring lesions, for which there are only limited treatment options. The life cycle of *L. mexicana* consists of two stages which involve different immune evasion mechanisms: promastigote and amastigote. Understanding parasitic interactions with host cells and developing a protective vaccine could improve the management and treatment of the disease.

Aims:

- To construct and compare the immunogenicity of 3 *Leishmania* genes in 3 different plasmids
- Study the effect of long term *in vitro* culture on the virulence of *L. mexicana* and its interaction with host cells
- To analyse the influence of *Leishmania* infection on MHC class I expression by susceptible human cell lines (U937 macrophages, U937 and MonoMac-6 monocytes), and Toll-like receptor, cytokines and chemokines gene expression profiles

Methodology: Three *Leishmania* genes (*L. mexicana* GP63, *L. donovani* centrin1 and *L. donovani* centrin3) were cloned into three plasmids (pcRT7/CT-TOPO; VR1012; and pcDNA3.1/Hygro(-)). The immunogenicity of the prepared DNA constructs and their empty counterparts was assessed in Balb/c mice using gene gun immunisation method. An *in vivo* model of attenuated *L. mexicana* was produced by growing the parasite *in vitro* for up to passage 20, and testing the infectivity of these parasites *in vivo* and *in vitro*. The influence of infecting target cells with virulent and avirulent *L. mexicana* at different growth stages on MHC class I expression was determined by flow cytometry. Gene expression profiles were determined by qPCR analysis of extracted mRNA.

Results: All tested *Leishmania* DNA constructs were highly immunogenic compared to the controls, as assessed using ELISPOT and cell proliferation assays. A novel survival assay developed in this study illustrated that macrophages derived from immunised mice were resistant to *Leishmania* infection. The parasite at passage 1 was highly infectious (virulent), but this progressively decreased to be completely avirulent at passage 20. This was associated with a significant down regulation of virulence-associated genes (GP63, LPG2, CPC, CPB2, CPB2.8, CHT1, LACK and LDCEN3) at passage 20, and was also accompanied by morphological changes. The avirulent parasite was unable to transform to the pathogenic amastigote stage in infected target cells. The gene expression profile of toll-like receptors (TLR-1, TLR-2, TLR-4, and TLR-9), cytokines (IL-1, IL-6, IL-10, IL-12 β , TNF- α , and TGF- β), and chemokines (CCL-1, CCL-2, CCL-3, CCL-4, CCL-5, and CCL-22) in target cells was induced and inhibited according to the virulence status of the parasite. Similar and significant down regulation of MHC class I was induced by infection of target cells for 24 hours with both virulent and avirulent *L. mexicana* parasite, however after 48 hours of infection only cells infected with avirulent but not virulent parasite have significantly restored their MHC class I expression.

Conclusions: Since no differences in the immunogenicity of the three plasmids encoding the same *Leishmania* gene were observed, immunogenicity is not dependent on the plasmid type. The failure of the avirulent *L. mexicana* parasite to infect Balb/c mice, and its inability to produce the pathogenic amastigote stage *in vitro* suggests that it might have potential as a vaccine candidate. This was also supported by the up regulation of Th2 mediators following the infection with virulent compared avirulent parasites. The level of MHC class I down regulation was dependent on parasite growth stage, virulence and infection dose.

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List of Abbreviations

ACTB	<i>Homo sapiens</i> actin, beta (ACTB), mRNA
AP-1	Activator Protein 1
APCs	Antigen Presenting Cells
B2M	<i>Homo sapiens</i> beta-2-microglobulin (B2M), mRNA
BM-DMs	Bone marrow derived macrophages
bp	Base pair
C3	Complement system component 3
C3b	Cleavage components of C3
CCL-1	<i>Homo sapiens</i> chemokine (C-C motif) ligand 1 (CCL1), mRNA
CCL-1	<i>Homo sapiens</i> chemokine (C-C motif) ligand 1 (CCL1), mRNA
CCL-2	<i>Homo sapiens</i> chemokine (C-C motif) ligand 2 (CCL2), mRNA
CCL-22	<i>Homo sapiens</i> chemokine (C-C motif) ligand 22 (CCL22), mRNA
CCL-22	Chemokine (C-C motif) ligand 22
CCL-3	<i>Homo sapiens</i> chemokine (C-C motif) ligand 3 (CCL3), mRNA
CCL-4	<i>Homo sapiens</i> chemokine (C-C motif) ligand 4 (CCL4), mRNA
CCL-5	<i>Homo sapiens</i> chemokine (C-C motif) ligand 5 (CCL5), mRNA
CCL-6	Chemokine (C-C motif) ligand 6
CCLs	Chemokines
CCLs	Chemokines
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
cDNA	Complementary deoxyribonucleic acid
DC	Dendritic cell
CFSE	Carboxyfluorescein succinimidyl ester
CL	Cutaneous Leishmaniasis
CPG	Cytosine-phosphate- guanine
CPs	Cysteine protease
CR	Complement receptors
CR3	Complement receptor3
CTL	Cytotoxic T lymphocyte
CXCL-10	C-X-C motif chemokine 10
CXCL-12	Chemokine CXC motif ligand 12
CXCL-8	C-X-C motif chemokine 8
CXCL-9	C-X-C motif chemokine 9
DCL	Diffuse cutaneous Leishmaniasis
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
dsRNA	Double stranded RNA
E	<i>Escherichia</i>
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FR	Fibronectin receptor
GAPDH	Human glyceraldehyde 3-phosphate dehydrogenase mRNA
GDP	Guanosine diphosphate
GIPL	Glycoinositolphospholipids
GP63	Metalloprotease-Glycoprotein of 63KD
HIV-1	Human immunodeficiency virus type 1
HSP70-II	heat-shock protein

List of Abbreviations

I.M	Intramuscular
iC3b	Inactive components of C3
IFN- γ	Gamma interferon
IgG	Immunoglobulin G
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
ILs	Cytokines
iNOS	Inducible nitric oxide synthase
JAK	Just Another Kinase
KARs	Killer activation receptors
L	<i>Leishmania</i>
L.dcen	<i>L. donovani</i> centrin (cen) gene, complete cds
L.mex LPG2	<i>L. mexicana</i> lpg2 gene
L.mex Cht1	<i>L. mexicana</i> chitinase (Cht1) gene, complete cds
L.mex CPB2	<i>L. mexicana</i> cpb2 gene
L.mex CPB2.8	<i>L. mexicana</i> lmcpb2.8 gene for cysteine proteinase
L.mex CPC	<i>L. mexicana</i> lmcpc gene cathepsin B-like cysteine proteinase
L.mex gp63-C1	<i>L. mexicana</i> GP63-C1 mRNA for metalloprotease gp63
L.mexgp63	<i>L. mexicana</i> GP63
L.mex- α tubulin	<i>L. mexicana</i> alpha tubulin gene
L.mex- β tubulin	<i>L. mexicana</i> beta tubulin gene
LACK	<i>Leishmania</i> homologue of receptor for activated C kinase
LCs	Langerhans' cells
Ldcen1	<i>L. donovani</i> centrin1
Ldcen3	<i>L. donovani</i> centrin3
LN	Lymph Nodes
LPG2	Lipophosphoglycan
LPS	Lipopolysaccharide
LYST	Lysosomal trafficking regulator gene
MARCKS	myristoylated alanine-rich C kinase
MARCKS	Myristoylated alanine-rich C-kinase substrate
MCL	Mucocutaneous Leishmaniasis
mDCs	Myeloid dendritic cells
MHC class I	Histocompatibility complex class I
MHC class II	Histocompatibility complex class II
MIP	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
NK cells	Natural killer cells
NK-k β	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
P	<i>Plasmodium</i>
PAMPs	Pathogen-associated molecular patterns
PBL	Peripheral blood leucocytes
PBS	Phosphate buffered saline
pDCs	Plasmacytoid dendritic cells

List of Abbreviations

PKC	Protein kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PMNs	Polymorphonuclear leucocytes
PTP	Protein tyrosine phosphatase
PTP1B	Protein tyrosine phosphatase subunit
PVs	Parasitophorous vacuoles
RNA	Ribonucleic acid
ROI	Reactive oxygen Intermediates
SHP-1	Protein tyrosine phosphatase subunit
SLA	Soluble <i>Leishmania</i> antigen
SLAM	Signalling lymphocyte activation molecule
ssRNA	Single stranded RNA
STAT1	Signal Transducer and Activator of Transcription
STAT4	Signal Transducer and Activator of Transcription protein family
T	<i>Trypanosoma</i>
TGF- β	Transforming growth factor beta
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR-1	<i>Homo sapiens</i> toll-like receptor 1 (TLR1)
TLR-2	<i>Homo sapiens</i> toll-like receptor 2 (TLR2)
TLR-4	<i>Homo sapiens</i> Toll-like receptor 4 (TLR4)
TLR-9	<i>Homo sapiens</i> toll-like receptor 9 (TLR9)
TLRs	Toll-like receptors
TNF- α	Tumor necrosis factor-alpha
VL	Visceral Leishmaniasis
WHO	World Health Organization

Chapter 1/ General introduction

1. General Introduction

1.1 *Leishmania* and Leishmaniasis

Leishmaniasis is a medical term used to describe complex symptoms that are caused by several types of an obligatory intracellular protozoan parasite belonging to the family Trypanosomatidae, genus *Leishmania* (Charmoy, 2010). Leishmaniasis is considered to be one of the two most important neglected endemic diseases (after Malaria) in tropical areas, (Bhowmick, *et. al.*, 2008). According to the World Health Organization (WHO), Leishmaniasis is currently epidemic in about 88 countries, over 12 million people are infected, 350 million are at the risk of infection worldwide and most of them are children (Olivier, *et al*, 2005). Thus, *Leishmania* spp infection is becoming a big concern for public health in the developing, as well as developed countries. The main challenge of *Leishmania* parasite infection is that, in spite of all attempts, an effective vaccine to prevent the spread of Leishmaniasis does not exist to date. Furthermore, Visceral Leishmaniasis has been reported as an opportunistic infection in HIV-1 patients across the globe (Mathur, *et. al.*, 2006), as illustrated in (Figure 1.1.1).

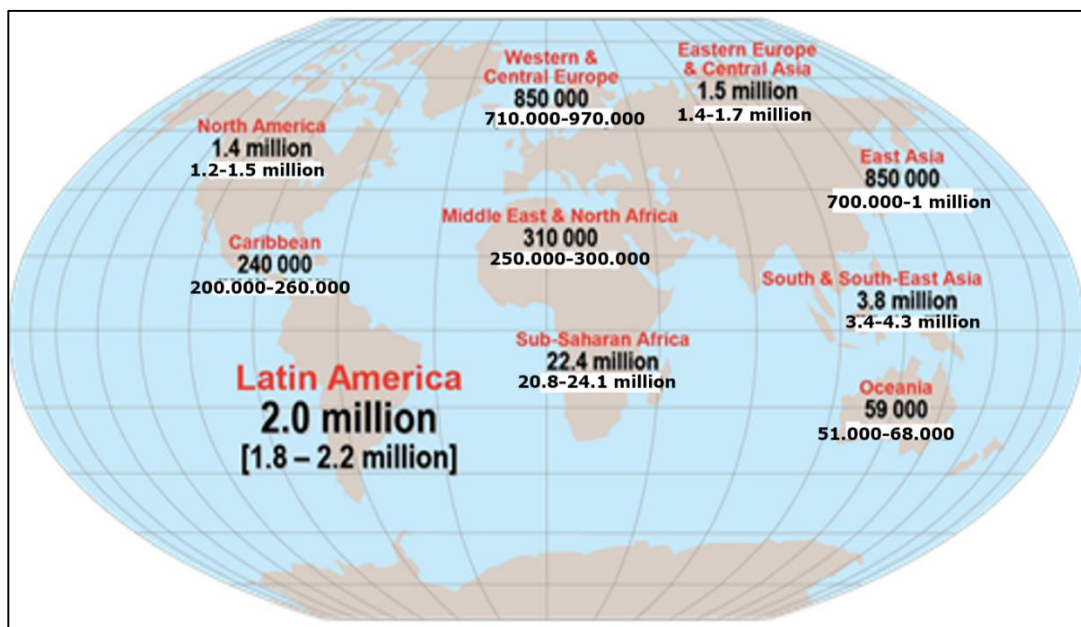


Figure 2.2-1 Visceral Leishmaniasis and HIV Distribution

The map illustrates the number of HIV individuals infected at different parts of the world (Adapted from Visceral Leishmaniasis-HIV co-infection: emerging in South-America) (<http://www.ectmihbarcelona2011.org/docs/Lindoso.pdf>)

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The disease is initiated by the bite of an infected female sand fly, which belong to Order of Diptera, Family of Psychodidae, subfamily Phlebotominae, and there are two genus belonging to this subfamily:

I) The genus *Phlebotomus* (Old World sand flies spp), which is found in the Mediterranean, Southern Europe, Africa, the Middle East, Central Asia, and South Asia.

II) The genus *Lutzomyia* (New World sand flies spp), which is found in the Americas. Interestingly, different species of sand fly females deliver different *Leishmania* species, as a consequence of which, different symptoms are developed (Ready, 2011).

1.2 Leishmaniasis history and *Leishmania* parasite life cycle

The history of Leishmaniasis is documented within two times, Old World Cutaneous Leishmaniasis and New World Cutaneous Leishmaniasis. The discovery of Old Cutaneous Leishmaniasis was reported in the first century anno domini (AD), whereas New Cutaneous Leishmaniasis was reported in the 16th century (Cox, 2002). Leishmaniasis was known as Kal-Zara in India, but the cause of this disease was unknown until the British scientist William Leishman developed the first Leishman's stain in 1901. Charles Donovan used this stain in 1903 to examine material from an infected patient. He found that the macrophages were heavily loaded with small organisms, which was the first indicator of the parasite infection. Later Donovan named these organisms (amastigotes) *L. donovani*. Nicolle and Comble following the *in vitro* incubation of samples from infected patients produced the second stage of the parasite life cycle (promastigotes). Col. Shortt and others confirmed the role of sand flies in *Leishmania* transmission in 1942 (Ul Bari, 2006). It has been well documented that the *Leishmania* parasite life cycle consists of two different morphological forms: a non-pathogenic form and pathogenic form, and these are found in different hosts: an invertebrate vector and mammalian host, respectively (Killick, 1990; Inverso, *et. al.*, 1993). The non-pathogenic form is an extracellular stage, and found in the mid-gut of *Phlebotomus* sand flies. This stage is a cylinder shape 10–20 µm in length known as a promastigote and is characterized by high motility due to the presence of a flagellum (Figure 1.2.1.A). In contrast, the pathogenic form is a rounded cell, and of 3-7 µm in diameter, called the amastigote stage (Figure 1.2.1.B). This is found in macrophages of mammalian hosts such as humans, rodents and dogs (Ashford, 2000; Cox, 2002).

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Infection of the mammalian host is initiated by the bite of an infected female sand fly, which delivers active metacyclic promastigotes into the host during blood feeding. Inside the mammalian host, the metacyclic promastigotes are taken up by various immune cells, usually antigen presenting cells such as macrophages and dendritic cells (DCs). Later, promastigotes are converted into the amastigote stage inside highly acidic compartments in host cells called parasitophorous vacuoles (PV). Amastigotes grow and multiply by binary fission, which eventually leads to cells rupturing and releasing free amastigotes to infect new macrophages. Therefore, whenever the non-infected female of a sand fly feeds on the infected mammalian host, infected macrophages are taken up with the blood meal and amastigotes are released inside the digestive tract due to the action of digestive enzymes. Free amastigotes travel back to attach themselves to the epithelium of the host midgut. The parasites at this stage rapidly multiply by binary fission and grow to procyclic promastigotes (Rogers *et al.*, 2002; Gossage, *et. al.*, 2003). After approximately seven days of blood meal feeding, the late stage (procyclic promastigotes) becomes less attached to the mid gut epithelium due to chemical changes in their surface molecules. This allows them to travel towards the front of digestive system. At this point, the parasites are transformed to metacyclic promastigotes, which are regurgitated into the bite site. This stage is categorised by a small cell body, long flagellum, free and rapid movement (Saraiva, *et. al.*, 1995), (Figure 1.2.2).

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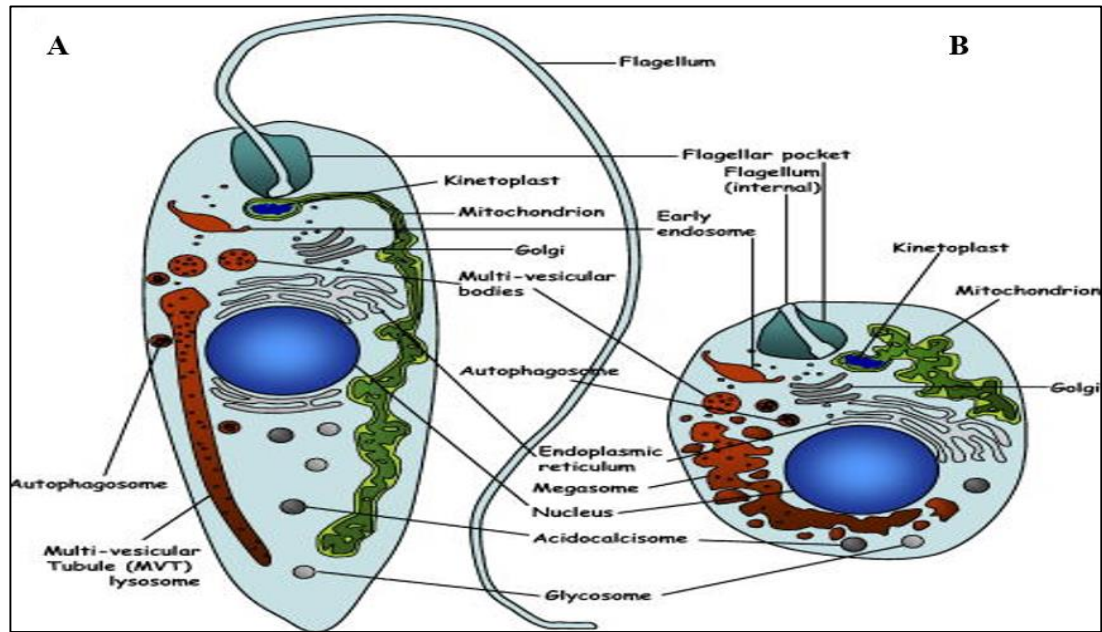


Figure 2.2-1 Diagram illustrating the *Leishmania* promastigote (A) and amastigote (B) (Morphology and intracellular organelles, adapted from Besteiro, *et. al.*, 2007) (Permission is not required for this type of reuse)

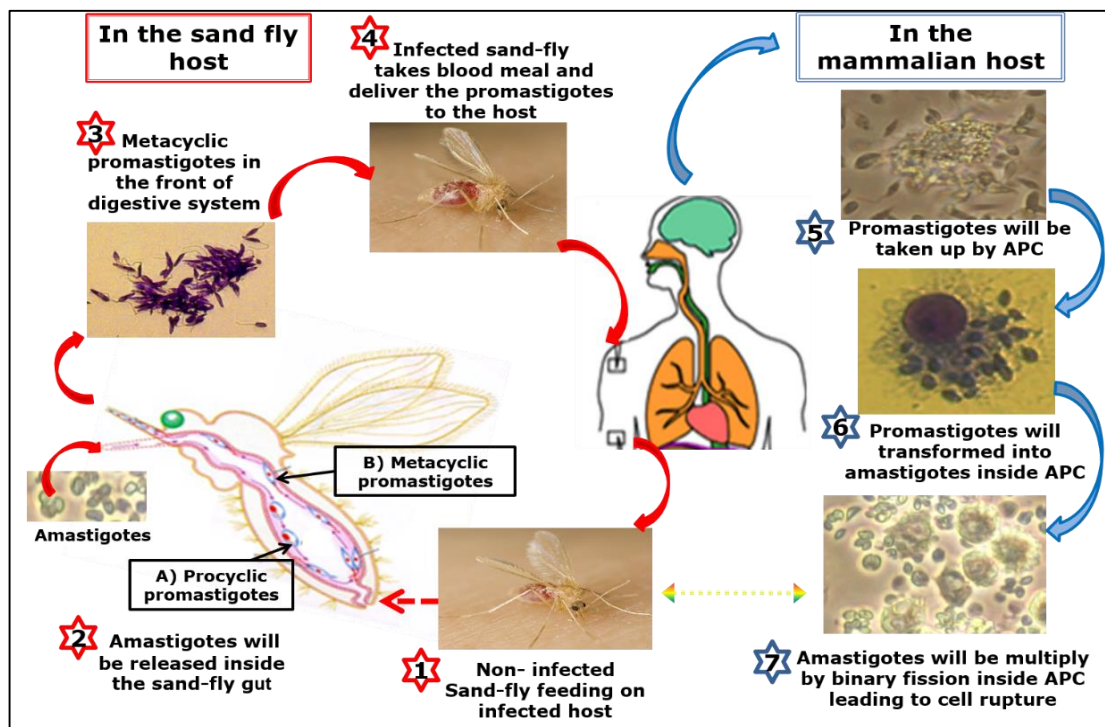


Figure 2.2-2 Diagram illustrating the *Leishmania* spp life cycle in two hosts

(1) Sand fly can be infected, following a blood meal from an infected mammalian host. Amastigotes in the midgut of the newly infected sand fly transformed into procyclic (A) and metacyclic (B) promastigotes to be cycled into a new host. In the mammalian host, promastigotes attack APCs and transform inside them into the pathogenic amastigote stage. This grows and multiplies by binary fission leading to host cell rupture and infection of more APCs. (Note: Parasite pictures in this diagram obtained from the current work).

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1.2.1 Leishmaniasis epidemiology

Leishmaniasis is a disease, which mostly associates with tropical and subtropical areas, where the environmental conditions are supporting the presence of the disease transmission vector. Leishmaniasis as previously mentioned is endemic in many areas, however, the global epidemiology of Leishmaniasis has not been sufficiently reported and the data is based on estimates in particular in the poor countries where most of the new cases exist.

For example, the recently published report by the WHO in 2013 has only shown the epidemiological map of Post-kala-azar dermal Leishmaniasis (PKDL), which is epidemic in East Africa, Sudan, South Sudan, Ethiopia, Bangladesh, India and Nepal (Figure 1.2.3). However, the report did not include statistics on other forms of Leishmaniasis. Other reports by WHO have stated that there were 638,702 cases of Cutaneous Leishmaniasis (CL) among Americans reported between 2001 and 2011, with an annual average of 58,063 new cases. In the same period, 38,808 cases of Visceral Leishmaniasis (VL), were also reported, with 96.6% of them in Brazil alone.

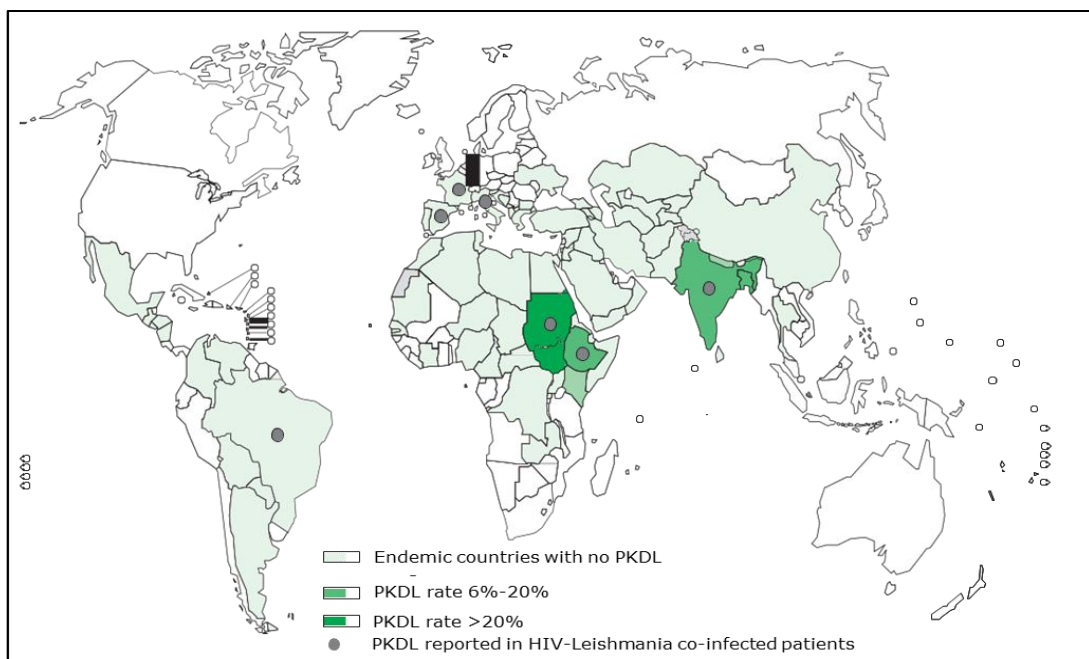


Figure 2.2-3 Global distribution of post-kala-azar dermal Leishmaniasis
(<http://www.who.int/leishmaniasis/resources/en/>)

1.2.2 Leishmaniasis transmission

One of the most important key players in the wide distribution of Leishmaniasis, is the spread of females *Phlebotomus* and *Lutzomyia* sand flies, in both Old and New worlds. These flies have been reported as vectors for the survival and development of the non-

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pathogenic form of *Leishmania* spp (promastigotes) and many viruses (Hendrick and Lancelot, 2010). In addition, they can transmit these organisms to the mammalian host during their blood meal. Recently, the problem of Leishmaniasis has increased, due to climate changes, which have affected the distribution of sand flies across the globe (González, *et. al.*, 2010). Of the 500 known sand fly species spread throughout the warmer climes around the world, just 31 species have been identified as vectors for *Leishmania* species (Killick, 1990). Interestingly, some species of sand flies can only transmit one species of *Leishmania* parasite. For instance, *P. Papatasi* (Figure 1.2-4 A) can only transmit *L. major*, whereas *P. Sergenti* (Figure 1.2-4 B) is known as a permissive vector, since it can transmit more than one species simultaneously. Adults of female sand flies are highly similar to each other, with small size 2-3mm, hairy body and conspicuous black eyes. Females of sand flies take a blood meal at a certain stage during their life cycle to lay their eggs in dark and humid places, so becoming a vector.

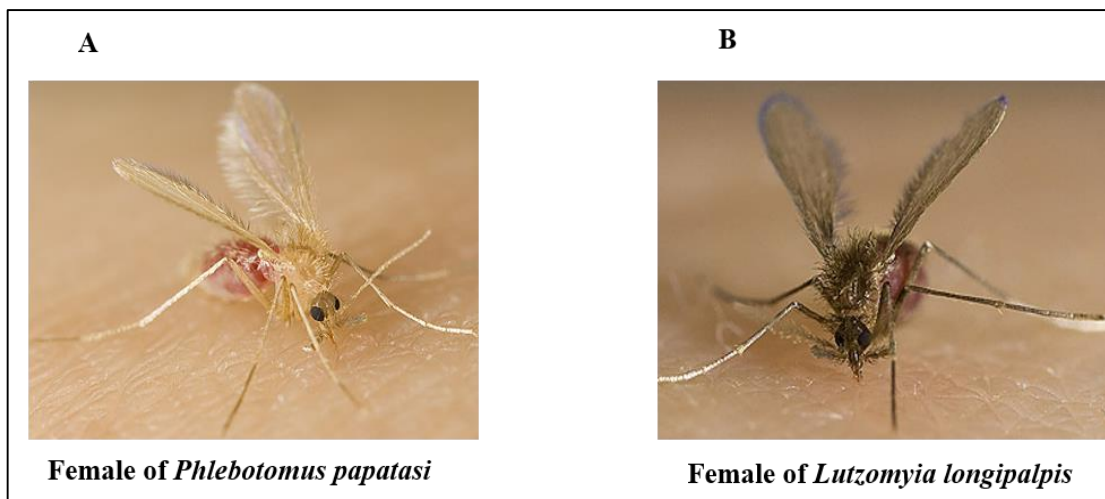


Figure 2.2-4 Different species of sand flies
(www.raywilsonbirdphotography.co.uk/Galleries)

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1.2.3 *Leishmania* parasite species

Leishmania is an obligatory intracellular pathogen, the final host of these parasites is usually APCs such as macrophages and DCs in various mammalian hosts such as humans, rodents and dogs. *Leishmania* parasites belong to the Order Kinetoplastida and Family Trypanosomatidae. This family was further sub-divided into sub families: *Leishmania* genus including species that complete their development in the anterior and midgut of the sand fly, and *Viannia* genus, which include the species that complete their development in the posterior gut of the vector (Lainson, *et. al.*, 1987). Due to the direct relationship between the clinical outcome of human diseases and the species causing the infection, the typical classification of *Leishmania* spp was originally based on the clinical forms of the disease (section 1.2.4). However, advanced techniques such as *Leishmania* genome sequencing and polymerase chain reaction (PCR) application are now widely used in *Leishmania* spp classification (Saravia, *et. al.*, 1985).

1.2.4 Clinical forms of Leishmaniasis

Depending on the state of the host immune system and its genetic background, the species of the *Leishmania* parasite and the place of final localization of the amastigote stage, the clinical symptoms of Leishmaniasis have been traditionally sub-divided into four main forms: Cutaneous Leishmaniasis (CL), Diffuse Cutaneous Leishmaniasis (DCL), Mucocutaneous Leishmaniasis (MCL) and Visceral Leishmaniasis (VL) (Etges, *et. al.*, 1986).

1.2.4.1 Cutaneous Leishmaniasis (CL)

Cutaneous Leishmaniasis (CL) is the most common and represents 50–75% of all new cases of Leishmaniasis (Mathur *et. al.*, 2006). The amastigotes of *Leishmania* parasite species are localized in the skin macrophages, consequently the CL was characterized by lesions in the skin at the site of the bite of an infected sand fly. People with CL develop lesions a few weeks after bitten by an infected sand fly. CL lesions usually heal spontaneously, leaving a depressed scar and long-lasting immunity (Minodier and Parola, 2007), although specific anti-*Leishmania* treatment is required form some lesion. CL is caused by several species of *Leishmania* such as *L. amazonensis*, *L. braziliensis*, *L. guyanensis*, *L. mexicana*, *L. panamensis*, *L. naiffi*, *L. venezuelensis*, *L. lainsoni*, and *L. shawi* in the New World. CL in the Old World is caused by *L. major*, *L. aethiopica*, *L. tropica* *L. arabica*, and *L. gerbilli* (Dowlati, 1996; Murray, *et. al.*, 2005) (Figure 1.2-5.A).

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1.2.4.2 Diffuse Cutaneous Leishmaniasis (DCL)

Diffuse Cutaneous Leishmaniasis (DCL) is caused the infection with *L. amazonensis* in the New World and *L. aethiopica* in the Old World (Etges, *et. al.*, 1986). DCL symptoms are characterised by many lesions at the site of a bite by an infected sand fly. It has been reported that DLC lesions cannot heal spontaneously (Weina, *et. al.*, 2004) (Figure 1.2-5.B).

1.2.4.3 Mucocutaneous Leishmaniasis (MCL)

The intracellular amastigotes in Mucocutaneous Leishmaniasis (MCL), are localized in the muco cutaneous membrane macrophages of the nose, mouth, ears and neck causing serious tissue disfigurement. MCL symptoms are developed mainly due to infection by *L. braziliensis* and *L. guyanensis* (Neva and Brown, 1994; Cox, 2002) (Finger 1.2-5.C).

1.2.4.4 Visceral Leishmaniasis (VL)

This type is the most severe form of Leishmaniasis and almost 100% fatal if untreated, since the amastigotes are localized in the spleen, liver and bone marrow. Patients with Visceral Leishmaniasis (VL) gradually develop symptoms of a systematic disease, such as irregular bouts of fever, substantial weight loss, splenomegaly and hepatomegaly and anemia, which eventually leading to the abdomen swelling. VL is caused by *Leishmania donovani* complex such as *L. donovani*, *L. infantum* and *L. chagasi* (Chappuis, 2007) (Figure 1.2-5.D).

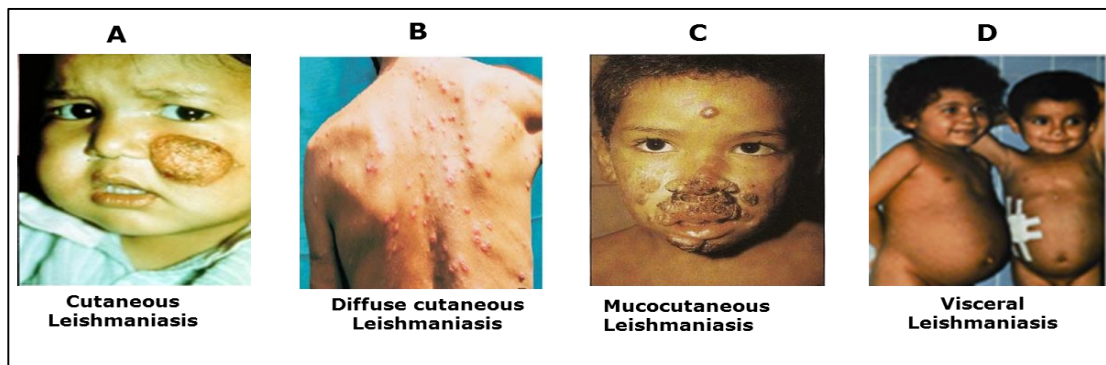


Figure 2.2-5 A, B, C and D. Leishmaniasis clinical symptoms

Based on the amastigote localization, clinical manifestations of Leishmaniasis can be divided into: **A** and **B**, Cutaneous Leishmaniasis and Diffused cutaneous Leishmaniasis (the amastigote survive in the skin macrophages). **C**, Mucocutaneous Leishmaniasis (the amastigotes survive in the muco cutaneous membrane macrophages). **D**, Visceral Leishmaniasis (the amastigotes survive in the spleen and the liver macrophages).

(http://dna.kdna.ucla.edu/168-2011/leish_files/leish%202012.pdf)

1.3 *Leishmania* promastigote surface molecules

Due to the challenges that the *Leishmania* parasite faces by being in two different hosts (invertebrate and mammalian, as promastigotes and amastigotes respectively), they develop a unique strategy to enable them both to survive in the sand-fly gut and subvert the mammalian host immunity. Many studies have reported that some proteins and surface molecules are over expressed during the infectious stage of the parasite (metacyclic promastigotes) compared to the non-infectious stage (procyclic promastigotes), and therefore they were considered virulent factors. In addition to their important role during the parasite life cycle, these proteins and molecules play an important role in parasite pathogenesis such as attachment, evasion and surviving inside APCs (Olivier, *et. al.*, 2012).

It is widely known that effective control of Leishmaniasis requires a good understanding of the parasite surface structure, which subsequently leads to identification of mechanisms used by the parasite to evade the immune system. Therefore, considerable attention has been paid to the structure and function of promastigote stage molecules, which can modulate and alter host cells mechanisms. Some of the well-known *Leishmania* spp surface molecules that are considered as virulence factors, and which have important roles in parasite pathogenicity, are discussed below.

1.3.1 *Leishmania* parasite Lipophosphoglycan

Lipophosphoglycan (LPG) is a main promastigote surface molecule that consists of a lipid backbone covered with disaccharide-phosphate components, linked to a glycan core (Turco and Descoteaux, 1992; Lodge and Descoteaux, 2005). The LPG molecules have multiple biological roles during the course of *Leishmania* parasite infection. For example, LPGs play a protective role in the promastigote against the induction of apoptosis by the complement system. Spath, *et. al.*, (2003) illustrated that LPG1^{-/-} *L. major* were susceptible to lysis by human complement lysis and vulnerable to the effects of oxidant reactions. This was in contrast to the findings by Ilg, (2000) who reported that the growth and survival of LPG^{-/-} *L. mexicana* promastigotes inside mouse macrophages was not affected. Another study reported the importance of LPG2 in *L. major* surviving in the sand-fly vector and mammalian hosts, as revealed by using LPG2^{-/-} *L. major* promastigotes (Svarovska, *et. al.*, 2010). An early study by Descoteaux and Matlashewski (1989) reported that *L. donovani* LPG has the ability to modulate the intracellular pathways such as protein kinase C (PKC) activity in infected

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macrophages. The role of LPG in PKC activity was further confirmed by Descoteaux and Turco (1999), who demonstrated that purified *L. donovani* LPG acts as protein inhibitors for PKC and blocks macrophage activation in response to LPS stimulation. Interestingly, the effect of LPG PKC activity in infected macrophage was cell type dependent. For example, *L. mexicana* LPG inhibited the PKC- α in macrophages derived from Balb/c mice, however, LPG purified from *L. mexicana* stimulated PKC activity in C57BL/6 macrophages (Delgado, *et. al.*, 2010). In addition, *Leishmania* promastigote LPG also contributes to immune evasion and differentiation into resistant amastigotes stage *via* inhibition of phagosome maturation. A study by Desjardins and Descoteaux, (1997) using LPG^{-/-} and wild type *L. donovani* promastigote to infect host cells, reported extensive phagosome-lysosome fusing in macrophages infected with mutant compared to wild type promastigotes. This phenomenon was further confirmed by using *L. major* in parallel with *L. donovani* knockdown LPG (where the target gene was deleted). Both strains were rapidly killed and removed by the infected macrophages. However, treatment with purified LPG prolonged their resistance (Descoteaux, Turco, 1999). Also, it has been shown that LPG interferes with nuclear translocation of NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) in monocytes which is facilitated by down regulation of IL-12 (interleukin-12) production (Argueta, *et. al.*, 2008), and activates the Th2 (T helper type 2) immune responses by up regulating of IL-4 (interleukin-4) production by DCs (Liu, *et. al.*, 2009).

Apart from its role as a virulence factor, LPG has an important role for the survival of the parasite inside the digestive system of the sand fly vector. The LPG structure of procyclic promastigotes (non-infective stage in mid-gut) is coated with more units of sucrose, which strengthens attachment of the parasite to the mid-gut. After being fully developed to metacyclic promastigotes (infective stage in the mid-gut), the structure of LPG is reformed with less units of sucrose in order to facilitate de-attachment of metacyclic promastigotes which travel to the front of the sand fly digestive system (Sacks, *et. al.*, 1995). In addition, LPG shows a capability to protect some *Leishmania* spp from sand fly digestive enzymes. For example, *L. donovani* strains deficient in LPG fail to finish their development in the vector (Sacks, *et. al.*, 2000).

1.3.2 *Leishmania* cysteine proteases

Cysteine proteases (CPs) belong to the *Leishmania* proteinases, which are considered as virulence factors due to their ability to hydrolyze peptide bonds in various protein structures, thereby causing considerable disruption of cell biology function (Silva, *et. al.*, 2012). In addition to the promastigote stage, CPs are also found in the amastigote stage (Coombs, 1982), and contribute to parasite transmission (hydrolyze the haemoglobin proteins), immune evasion and inflammation response at the site of infection (Sajid and Mckerrrow, 2000; Mckerrrow, *et. al.*, 2006).

This group of enzymes was thoroughly investigated in *L. mexicana* in the early 1980s, and it was sub-divided into three main groups according to biochemical characteristics (Robertson and Coombs, 1994):

- First group: type I cysteine protease such as CPB genes, which are also found in *Trypanosoma* spp (Mundodi, *et. al.*, 2002).
- Second group: type II cysteine proteases, the biological function of the second group is not fully understood (Rafati, *et. al.* 2001).
- Third group: type III cysteine proteases such as CPC gene (Robertson and Coombs, 1994).

Unlike other *Leishmania* spp, the biological function of CPs was well investigated in the *L. mexicana* complex (Mottram, *et. al.*, 1997; Mottram, *et. al.*, 2004), *L. pifano* (Bryson, *et. al.*, 2009) and *L. amazonensis* (Lasakosvitsch, *et. al.*, 2003). The biological function of CPs was illustrated by knocking (the target gene was removed) parasite CPs genes. For example, a study using *L. mexicana* CPA, CPB and CPC knockout showed that lesions in Balb/c mice infected with parasite knockout CPB were significantly smaller and less in number compared to the mice injected with parasites CPA or CPC genes knockout (Jardim, *et. al.*, 1995; Alexander, *et. al.*, 1998). Interestingly, the restoration of the CPB gene did not completely recover the parasite virulence, while restoration of multiple copies of CPs genes did so. These findings revealed that multiple copies of CPs genes are required for the function of these enzymes. The failure of *L. mexicana* promastigotes CPB knockout to produce full infection in inoculated Balb/c mice correlated with the failure of the parasite to mount a Th2 immune response, which is associated with IL-4 production (Denise, *et. al.*, 2003).

Immune responses to parasite infection generally depend on the mice strain. For example, infection in C57BL/6 resistant mice was associated with the down regulation

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of T helper type 1 (Th1) related cytokines, while disease progression in Balb/c mice was associated with the generation of Th2 cytokines such as IL-4 (Buxbaum, *et. al.*, 2003).

CPB can also inhibit target cell signalling pathways such as NF- κ B, AP-1 and STAT1, leading to the inhibition of IL-12 production by infected macrophages (Cameron, *et. al.*, 2004). This was confirmed when *L. mexicana* CPB knockout failed to inhibit NF- κ B. In addition, CPBs of *L. amazonensis* were reported to prevent antigen presentation *via* MHC II on the surface of PVs (Courret, *et. al.*, 2001), where the ability of *L. amazonensis* amastigotes to degrade MHC II on the PV surface of their host was demonstrated. Although the role of CPB in the virulence of the *L. mexicana* complex has been well characterised compared to the *L. donovani* complex, CPs activity in *L. braziliensis* (the main cause of CL in the New World) has not yet been well defined. Non-CPB cysteine proteinases such as CPC and CPA have also been found to contribute to the virulence of *L. mexicana* complex. For example, *in vitro* experiments illustrated that *L. mexicana* amastigote CPC knockout was able to infect macrophages similarly to wild type amastigotes, but that amastigotes were more susceptible to killing by infected macrophages (Mottram, *et. al.*, 1997). Somanna, *et. al.*, (2002), reported the ability CPC of *L. chagasi* (belonging to the *L. donovani* complex) to suppress the immune response by up regulating TGF- β expression in infected human cell lines. Although it has been reported above, the infectivity of *L. mexicana* promastigotes CPA knockout was as effective in Balb/c mice as wild type promastigotes (Alexander, *et. al.*, 1998). Nevertheless, the growth and infectivity of *L. mexicana* promastigote in which both CPA and CPB genes had been knocked out was inhibited more compared to the growth and infectivity of parasites when only one gene was knocked out (Lasakosvitsch, *et. al.*, 2003).

1.3.3 *Leishmania* parasite metalloprotease GP63

A considerable amount of attention has been paid to the zinc-dependent metalloprotease or glycoprotein gp63 (GP63) molecule as an important virulence factor in the past few decades (Olivier, *et. al.*, 2012), due to its importance in the immune escape mechanisms used by *Leishmania* parasite. In addition to its expression in promastigotes, GP63 protein is also expressed in the amastigote stage across known *Leishmania* spp (Yao, *et. al.*, 2003). This protein (GP63) has a range of activities that significantly increases the severity of parasite infection. The importance of GP63 as a

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virulence factor can be seen in the first few hours of interaction between the promastigotes and host cells, through the inhibition of the complement mediated lysis through changing the active form of C3b into the iC3b inactive stage (Brittingham, *et. al.*, 1995). Cleavage of C3b to iC3b by GP63 does not only fix and reduce the complement lytic activity, but also enhances promastigote entrance *via* C1 and C3 complement receptors (Mosser and Edelson, 1985). McGwire, *et. al.*, (2003) reported another biological role for promastigote GP63 by showing that an extracellular matrix of subcutaneous tissue could be degraded by *L. mexicana* promastigotes GP63. This favoured the rapid migration of the host cells to the site of infection, thus increasing the possibility of promastigote host cell-entrance.

GP63 also has an important role in the differentiation and maintenance of the intercellular amastigote stage by targeting and modulating the host cell signalling mechanisms (Halle, *et. al.*, 2009). For example, *Leishmania* GP63 can interfere with other biological signalling pathways, such as the myristoylated alanine-rich C kinase (MARCKS) pathway, which is an important PKC substrate in macrophage responses (Corradin, *et. al.*, 1999). Furthermore, GP63 can modulate the protein tyrosine phosphatase SHP-1 activation that is required for the IAK2/STAT1- α pathway and IFN- γ -NO (interferon gamma-nitric oxide) production (Shio, *et. al.*, 2012). It is worth mentioning here that GP63 protein is not only attached to the surface of promastigotes, but can be also found as a secreted form (Silverman, *et. al.*, 2010), as many studies have reported the modulation and interference of secreted proteins with the infected host cells. The GP63 exosomes in *L. mexicana* can be released into the cytoplasm of infected cells under the effect of host temperature. These exosomes are able to block NO secretion (Hassaani, *et. al.*, 2011). A similar finding has been reported for *L. donovani* exosomes, which induce IL-10 (Interleukin-10) and TFN- α (tumor necrosis factor) production, and inhibited IFN- γ secretion (Silverman, *et. al.*, 2010).

Although the biological role of GP63 in the promastigote stage has been extensively investigated, there are still debates about its biological role in the survival of the amastigote stage across *Leishmania* spp. Nevertheless, Chen, *et. al.*, (2000) demonstrated that down regulation of GP63 in *L. amazonensis* promastigotes reduces their survival inside infected macrophages, which may indirectly illustrate the role of GP63 in maintaining of amastigote stage. An early study by Chaudhuri, *et. al.* (1989), reported that proteins coated with *L. mexicana* GP63 were protected from

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phagolysosome degradation, thereby illustrating the protective role of GP63 to coated organisms. This was further supported by the work of Seay, *et. al.*, (1996) who demonstrated that attenuated *L. mexicana amazonensis* has a low survival rate compared to virulent *L. mexicana amazonensis* correlated with a 20-30 fold down regulation of GP63.

1.4 Immunity to *Leishmania* parasite infection

The innate and adaptive immune responses to infection vary according to the pathogen type, consequently some of these pathogens would be killed by just the innate immune response whereas others require activation of adaptive immune responses. However, some pathogens can avoid and/or escape immune defence lines and cause major health problems.

Several studies have established that the immune response to *Leishmania* infection relies on different factors. Some of these factors are related to the mammalian host and its genetic background, other factors are related to the parasite species that cause the infection (Okwor and Uzonna, 2009; Birnbaum and Craft, 2011). In addition, species of the female sand fly genera *Phlebotomine* and *Lutzomyia*, which transmit the infection to the mammalian host, may contribute to the exacerbation of the *Leishmania* infection (Palatnik, 2008).

Over evolutionary time, *Leishmania* parasite species, have developed unique mechanisms that favour their survival inside the host immune system. These parasites can successfully evade and exploit immune defences by being intracellular antigens. Studies have illustrated that intracellular amastigotes were more resistant to immunity compared to the extracellular promastigote stage. After delivery to the host skin by either natural or experimental transmission, *Leishmania* parasites follow highly specific strategies in order to establish their survival in the new host, as reflected by the extent of Leishmaniasis symptoms (Mendez, *et. al.*, 2001; Chang and Mcgwire, 2002; Olivier, *et. al.*, 2005).

1.4.1 Innate immune response to *Leishmania* infection

Though the innate immune is the first defence following antigen entrance, the mechanisms by which this system works are poorly understood in comparison to the adaptive immune response (Parham, 2009). In *Leishmania* infection, almost all of the known components of the innate immune response such as complement system, leukocytes (monocytes, macrophage, neutrophils, and DCs), and natural killer (NK) cells, contribute to the defence against this parasite. Interestingly, the *Leishmania* parasite can be selective of the target cells that may support their immune evasion. For instance, *L. major* promastigotes were found in skin fibroblasts, whereas other *Leishmania* spp are found in stromal cells (Bogdan, *et. al.*, 2000). This preference was first explained as stromal cells providing a safe environment for the parasite's survival.

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It was later reported that infection of particular cells by the *Leishmania* parasite is an immune evasion mechanism. Svensson, *et. al.*, (2004), demonstrated that CCL-1 and CXCL-12 chemokines were up regulated in spleen stromal cells infected with *L. donovani* and that they recruited dermal DCs to the site of parasite infection, which inhibits the Th1 response. However, a comprehensive understanding of the interaction between the *Leishmania* parasite and various host cells is an extremely important for following the development of approaches for controlling Leishmaniasis.

1.4.1.1 Toll-Like Receptor and *Leishmania* parasite infection

Although the immune system sometimes fails to control the infection, nevertheless, innate immune components are still able to recognise and interact with the microbes, using different strategies. One of the well-established molecules used by the innate immune system to recognize foreign antigens, including *Leishmania* infection, is a group of proteins called Toll-Like Receptors (TLRs) (Tuno, *et. al.*, 2008). The TLRs can recognize a wide range of biochemical markers on the pathogen's surface called pathogen-associated molecular patterns (PAMPs). It is known that in humans there are 10 toll-like receptor genes, each of which specific for a different set of pathogenic products (Parham, 2009). The interaction of TLRs with PAMPs leads to activation of related cells such as monocytes, macrophages, neutrophils, DC, and NK cells (Tuno, *et. al.*, 2008). Receptor ligation also trigger the secretion of cytokines from these cells (Akira, *et. al.*, 2001; Medzhitov, 2001; Dabbagh and Lewis, 2003).

The role of TLRs in infection control of many pathogens has been extensively investigated (Medzhitov, 2001). Although TLRs are classified as molecules of the non-specific immune response, different TLRs are usually activated by different antigens which related to different pathogens such as bacterial DNA, bacterial endotoxin (LPS), virus double stranded RNA (dsRNA) and species of single stranded RNA (ssRNA) (Dempsey, *et. al.*, 2003). TLRs are located inside the cells (on lysosome membranes such as TLR-3, TLR-7, TLR-8 and TLR-9), and/or outside the cells (on the cytoplasm membrane such as TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6) (Tuno, *et. al.*, 2008). Both stages of *Leishmania* spp have the chance to interact with TLRs and modulate the initial immune response, which favours their survival inside the host cells. Although recognition of the antigens is initiated by the TLRs, the immune response is achieved *via* intracellular activation of NF- κ B pathway through myeloid differentiation factor 88 (MyD88). Briefly, recognition of extracellular antigens like LPS by TLR-4 leads to

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intracellular binding of the TIR domain of TLR-4 with the TIR domain of MyD88 protein, this complex is acting as a substrate to start a series of other intracellular reactions, which mostly rely on the phosphorylation of protein kinase C (PKC). This process is ended by the degradation of the I κ B inhibitor which releases the NF- κ B to travel to the nucleus and bind to the DNA to start gene transcription of inflammatory cytokine (Peter, 2009).

The importance of TLRs and the MyD88 pathway towards *Leishmania* parasite control was first demonstrated *in vitro* by Hawn, *et. al.*, (2002) using MyD88^{-/-} macrophages. Their results showed a down regulation of IL-1 expression in macrophages infected with *L. major* compared to IL-1 expression in MyD88^{+/+} macrophages. Another study has illustrated that MyD88^{-/-} C57B/6 mice were susceptible to *L. major* infection, compared to wild type C57BL/6 mice (Muraille, *et. al.*, 2003).

It has been found that the role of TLRs contribution to *Leishmania* parasite infection is different according to which TLR ligand is involved in recognition of the parasite antigen. For example, the role of TLR-4 in *L. major* control through NO production was investigated using knockout TLR-4 (TLR-4^{-/-}) knockout C57BL/6 mice. The result showed that the infection control in wild type C57BL/c mice was achieved *via* enhancement of NO production in infected cells. In contrast, susceptibility of TLR-4^{-/-} C57BL/6 mice to *L. major* infection was associated with decrease in NO production, and the infection was controlled after treatment of infected mice with IL-12 (Kropf, *et. al.*, 2004; De veer, *et. al.*, 2003). Furthermore, C57BL/6 macrophages that had been knocked out for the expression of the signalling lymphocyte activation molecule (SLAM), which has an important role in TLR-4 activation, were more susceptible to *L. major* infection compared to their wild type counterparts (Wang, *et. al.*, 2004). Although TLR-4 contributes to infection control, TLR-2 was found to exacerbate the *Leishmania* parasite infection. Vargas, *et. al.*, (2009) reported that TLR-2 was required for *L. braziliensis* infection and also that the lack of TLR-2 enhanced the maturation of DCs and resulted in a high production of IL-12. This was further confirmed using TLR-2 deficient C57BL/6 mice which were more resistant to *L. amazonensis* infection compared to the wild type C57BL/6 mice (Guerra, *et. al.*, 2010).

1.4.1.2 Chemokine and *Leishmania* parasite infection

Infection control and pathogen clearance require a strong immune signal to enable the recruitment of immune cells to the site of infection. This process is mediated by a group

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of small proteins (8-10 kilo Dalton) (Oghumu, *et. al.*, 2010) called chemokines which behave as chemo-attractants to direct cell movement (Teixeira, *et. al.*, 2006). Gordon (2002) has reported that there is a direct relationship between cytokines and chemokine secretion, in that cytokine production leads to the up regulation of chemokine gene expression or production. For example, the secretion of IFN- γ controls the production of CXCL-9 and CXCL-10 chemokines (Farber, 1997). The secretion of related Th2 cytokines (IL-4 and IL-13) induces the production of CCL-22 and CCL-6 from infected macrophages, and this production can be inhibited by IFN- γ (Bonecchi, *et. al.*, 1998; Orlofsky, *et. al.*, 2000).

Many studies have reported on the importance of the chemokines in *Leishmania* infection control *via* up regulation of the gene expression profile of chemokines related to Th1 immune responses (Ritter and Korner, 2002; Antoniazzi *et. al.*, 2004). Th1 related chemokines (CCL-2, CXCL-9 and CXCL-10) are highly produced in self-healed CL lesions induced by infection with *L. mexicana*. In contrast, a high production of Th2 related chemokines such as CCL-3 has been found to be associated with DCL (Ritter and Korner, 2002). Arnoldi and Moll (1998) demonstrated the role of CCL-3 chemokine (also known as macrophage inflammatory protein, (MIP) in murine CL for the transport of the *Leishmania* parasite from the infected skin to lymph nodes (LNs) by activated Langerhans cells. The role of CCL-2 as a Th1 chemokine was demonstrated *via* its capability to induce anti-*Leishmania* activity by increasing the respiratory burst in infected monocytes (Ritter and Moll, 2000).

Due to the high immunogenicity of infected sand fly saliva (Sacks and Kamhawi, 2001) PMNs are the first cells to arrive at the site of infection. These cells secrete the CXCL-8 chemokine (also known as IL-8) (Muller, *et. al.*, 2001; Laufs, *et. al.*, 2002), which attracts more cells to the infection site. Although the CXCL-9 and CXCL-10 chemokines are involved in the control of *Leishmania* infection in CL, their detection at a high concentration in the serum of patients with VL (Hailu, *et. al.*, 2004), may suggest a role for them in disease progression.

1.4.1.3 Soluble immune responses to the *Leishmania* spp infection

Nitric oxide (NO) activity is one of the well-known microbicidal mechanisms contributing to parasite clearance, and levels of this are controlled by the expression of inducible nitric oxide synthase (iNOS) in macrophages (Huang, *et. al.*, 2011). The NO, which kills the parasite, is produced by the enzymatic oxidation of the guanidine group

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of L-arginine, which is induced by Th1 immune responses *via* IFN- γ production (Töttemeyer, *et. al.*, 2006). Th2 immune response-related cytokines (IL-4, IL-13 and IL-10) inhibit the production of NO by producing the arginase enzyme, which competes with iNOS for the same substrate (Brittingham, *et. al.*, 1990). The role of NO in parasite clearance was clearly demonstrated by infection of mice knocked out for iNOS (Wei, *et. al.*, 1995). These mice showed an inability to control infection by NO production. Non-specific enzymes are secreted during an antigen phagocytosis process by active macrophages. Therefore, these enzymes are also having a major impact on killing foreign organisms including *Leishmania* parasites (Mink, *et. al.*, 2009).

Experimental modelling shows the need for physical interaction between the parasite and macrophages for NO production, since macrophages incubated with *Leishmania* parasite molecules (LPG and GIPL) show no effect on the production of NO, even after stimulation of target cells with IFN- γ or LPS, when compared to macrophages infected with whole parasites (Proudfoot, *et. al.*, 1995; Proudfoot, *et. al.*, 1996). It has been well established that NO has a unique function for parasite clearance. This role was illustrated by Diaz, *et. al.*, (2003) who reported that NO levels in the sera of either susceptible Balb/c mice or resistant C57BL/6 mice infected with *L. mexicana* was different in both strains. Their results clearly showed the decrease of NO in the serum of infected Balb/c mice was due to the lack of iNOS in susceptible Balb/c macrophages.

1.4.1.4 Dendritic cells and *Leishmania* parasite infection

Dendritic cells (DCs) are group of cells that circulate in the blood in their immature state or presented in the tissue such as lymph nodes, where they provide the opportunity for antigen recognition to occur *via* T-cell receptors (Bousso, 2008). These cells after being in contact with the antigens become mature and are considered as professional APCs. Typically, DCs are classified into three subsets according to their source and surface markers in humans:

- Myeloid DCs (mDC1): this group is CD11c+, and they circulate in the blood.
- Plasmacytoid DCs (pDC2): this group is CD11c- and is mostly found in the lymph organs.
- Langerhans' cells (LCs): this group is CD205, CD11b^{low}, MHC II^{high} and are found in the skin (Brandonisio, *et. al.*, 2004; Soong, 2008).

DCs are characterised by their movement due to blood circulation compared to other APCs such as macrophages. Thus, DCs have unique biological roles in the immune

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response, where they take up foreign antigens from the site of infection and migrate to the lymph organs to present the degraded antigen to naïve T cells *via* the histocompatibility complex (MHC) peptide complex. Studies have reported that the responsiveness of DCs to *Leishmania* spp varies according to DC subtype. For example, *in vitro* infection with different *Leishmania* spp has shown that mDCs were more effective and able to engulf the promastigotes compared to LCs (Von, *et. al.*, 2000). Interestingly, parasite infection has been shown to modify the expression of surface antigens by DCs, and also their secretion of cytokines and chemokines. Matured DCs are recognized by some criteria such as CD40, CD80, CD86, and CD54, as well as the high expression of toll-like and chemokine receptors (Brandonisio, *et. al.*, 2004). *Leishmania* spp modulate the expression of matured DCs markers, which enable them to be safely transported around the body. For example, infection of Balb/c mice DCs with *L. mexicana* has down regulated the express of CD40, CD80, and CD205 DCs markers (Astial, 2011). One of the most important proinflammatory cytokines in parasite clearance is interleuken-12 (IL-12), and the main source for this cytokine following the initial interaction with the promastigotes is DCs (Konecny, *et. al.*, 1999). It has been reported that infection with different *Leishmania* spp have various effects on the secretion of IL-12 from DCs. For example, *in vitro* infection of murine mDCs with *L. mexicana* promastigotes has blocked IL-12 production and DCs maturation (Bennett, *et. al.*, 2001). However, infection of murine pDCs with *L. major* promastigotes caused IL-12 production (Henri, *et. al.*, 2002). IL-12 secretion from human mDCs was inhibited following infection with *L. donovani* and *L. tropica*, but not by *L. major* promastigotes (McDowell, *et. al.*, 2002).

Infection with some *Leishmania* spp can arrest the migration of the DCs. For example, Jebbari, *et. al.*, (2002), illustrated that LPG from *L. major* promastigotes has a negative effect on the migration of pDCs and LCs from the site of infection to the lymph organs. Other species such as *L. amazonensis* can delay the maturation of mDCs (Prina, *et. al.*, 2004), thus they will have enough time to produce the amastigote stage inside infected cells.

1.4.1.5 Natural killer and *Leishmania* parasite infection

Natural killer (NK) cells are generated in the bone marrow from the lymphoid progenitor cell lineage that gives rise to B and T cells. NK cells are released into the blood, and placed finally in different tissues including the circulatory system, where they comprise

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5-15% of total lymphocytes. NK cells are characterized by their larger size and granular cytoplasm when compared with other lymphocytes. These cells have unique cell surface molecules, which enable them effectively to carry out their biological role. NK cells are recognized by their expression of CD16, CD56, killer activation receptors (KARs), killer inhibition receptors (KIRs), FcγRIII which mediate the antibody-dependent cellular cytotoxicity (ADCC) and TLRs which take part in cytokines and cytotoxicity production (Tosi, 2005). In addition, they do not have the natural killer T cells (NKT) markers, which are CD3 and TLR.

The biological role of NK cells is to destroy pathogenic infected cells including some tumour cells. Infected or abnormal cells would be recognized by NK cells through their surface KAR molecules, which induce NK-cell killing. However, normal cells are protected from NK killing mechanisms by their surface KIRs molecules, which inhibit and induce a negative signal to NK cell killing mechanisms, these receptors can recognize self MHC I-peptide. Therefore, some pathogens such as viruses and parasites down regulate the expression of MHC molecules in infected cells so infected cells would be less recognized by cytotoxic T cells, but will make them more susceptible to NK killing. The mechanism of NK killing is performed by the release of perforins and granzymes onto the surface of infected cells. Perforins increase the permeability infected cells, and allows the internalisation of proteolytic enzyme (granzymes) thereby leading eventually to cell apoptosis. Unlike virus infections, NK cells do not have a direct role in parasite infection control, however, they can induce *Leishmania* cells death by infected macrophages through their secreted cytokines such as IFN-γ, and IL-12, which promote a Th1 immune response (Prajeeth, *et. al.*, 2011).

1.4.1.6 The interaction between *Leishmania* spp and macrophages

Metacyclic promastigotes are directly injected into the host by infected females of sand flies, and the establishment of intracellular amastigotes is the main challenge facing the first entrance of promastigotes to the host. As soon as the metacyclic promastigotes enter the host, they would be taken up by APCs. Although *in vitro* studies have shown that macrophages are usually the most important APCs during *Leishmania* infection, other studies have reported that neutrophils quickly engulf the metacyclic promastigotes, and that these survive inside their phagosomes (Kaye and Scott, 2011). Infected neutrophils would be triggered into self-death by apoptosis, and targeted by macrophage engulfment, where they become target antigens for the macrophages.

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These antigens in turn enter the macrophages in a silent phase, leading to the successful amastigote transformation (Ravichandran and Lorenz, 2007). Use of two-photo intravital imaging has given a clear picture of the role of neutrophils following needle inoculation or sand fly transmission of *L. major* to mice model (Peters, *et. al.*, 2008). Neutrophils have a similar role following infection with *L. infantum*. However, the depletion of neutrophils at the site of *L. braziliensis* infection increased mice susceptibility (Novais, *et. al.*, 2009; Thalhoffer, *et. al.*, 2011).

Macrophages are effectively capable of engulfing metacyclic promastigotes injected by infected sand fly or needle inoculation into their phagosomes. Different *Leishmania* spp have developed different mechanisms to either activate opsonisation-independent phagocytosis (which results a safe host cell entrance), or inhibit the opsonisation-dependent phagocytosis (caused by the complement system or the immunoglobulin proteins) (Horta, *et. al.*, 2012).

An engulfed parasite is finally arrested inside macrophage organelles called parasitophorous vacuole (PVs), in which the metacyclic promastigotes can complete their differentiation into the amastigote stage. PVs are well known to be a safe environment for parasite growth and multiplication away from host immune responses, as the biology and function of macrophages is highly affected after *Leishmania* infection. Moreover, studies have reported that PVs usually have a highly acidic condition and also have various lysosome enzymes including proteases (Candolfi, *et. al.*, 1994). In addition, PV organelles have been found to contain major histocompatibility complex (MHC) class II molecules but not MHC class I molecules, after their maturation with IFN- γ (Candolfi, *et. al.*, 1994; Charmoy, 2010).

One of the major effects of the phagocytosis of *Leishmania* by macrophages is the modulation of several mechanisms and signalling pathways of infected macrophages. For example, *Leishmania* parasites can partly subvert the immune response and thus support survival inside the host by modulation of macrophage cytokines production (Kane and Mosser, 2000; Cunningham, 2002). Although IL-12 is one of the most important cytokines following the infection of macrophages, IL-12 production is subsequently followed by activation of the Th1 response and production of IFN- γ (Piedrafita, *et. al.*, 1999; Teixeira, *et. al.*, 2006). Many studies have reported the down regulation of IL-12 following *Leishmania* infection (Cunningham, 2002; Kane and Mosser, 2000). Moreover, infection with *Leishmania* parasites up regulates the

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secretion of anti-inflammatory (IL-10 as well as IL-4) and inhibits the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α and IL-1 (Belkaid, *et. al.*, 2001; Cunningham, 2002).

In addition to their essential role in the innate immune response, macrophages are important in the adaptive immune response *via* activation of cell-mediated immune responses. Thus, macrophages are not only the host for the promastigotes, but are also effective cells that may kill engulfed promastigotes. Therefore, the occurrence of *Leishmania* infection depends on the balance between the ability of macrophages to kill and the parasite capable to suppress and evade the immune response.

Early studies on gene expression profiling of non-infected and *L. donovani* infected Balb/c mice macrophages illustrated that only 40% of several hundred tested genes by microarrays were down regulated after infection of macrophages for 4 days (Chang, *et. al.*, 2003). Since Leishmaniasis is caused by infection with different *Leishmania* spp, the mechanisms used by these parasites to escape immune host defence may also vary. When Gregory, *et. al.*, 2008 applied the microarray technique to assess the gene expression profiles of bone marrow-derived macrophages using *L. donovani* and *L. major* infection, they found no significant difference between these two species on the macrophage gene expression profile, even though they caused different clinical diseases symptoms.

Since the macrophages normally express MHC class I and MHC class II molecules, they are capable of presenting motifs from degraded foreign antigens on their surface to be seen and recognized by other immune cells, in particular naive T cells (Parham, 2009). However, the biggest challenge in immunity to Leishmaniasis is that the *Leishmania* parasite can be latent for years inside host macrophage.

1.4.1.7 Inhibition of phagosome maturation in *Leishmania* parasite infection

As mentioned above, the PVs are a permissive compartment for amastigote transformation and proliferation inside host cells including neutrophils, macrophages, DCs and fibroblasts (Bogdan, *et. al.*, 2000). It has been demonstrated that PVs have the properties of endosomes-phagosome, including the expression of specific membrane markers (Morehead, *et. al.*, 2002). In addition, as PVs have different sizes and they vary in their morphology, they can hold different amastigote numbers according to the *Leishmania* species and host cell type. For example, species like *L. amazonensis* and *L.*

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mexicana survive in big PVs, which contain many amastigotes compared to *L. major* and *L. donovani*, which produce small PVs with a single amastigote (Wilson, *et. al.*, 2008). In extracellular infections, macrophages engulf the antigens inside their phagosome which later fuses with host cell lysosomes leading to complete degradation of the foreign engulfed antigen or microbes and their expression on MHC molecules. The process of phagosome-lysosome fusion is referred to as phagosome maturation (Scharton and Scott, 1993). *Leishmania* species have a unique ability to delay and modulate the phagosome-lysosome, thus they would be prevented from being presented to adaptive immunity. Phagosome maturation delay after *Leishmania* spp infection was first reported by Desjardins and Descoteaux (1997) following infection of macrophages with *L. donovani* due to the presence of LPG molecules. Infection of transgenic mice Rab5 positive bone marrow derived macrophages with *L. mexicana* promastigotes has led to a similar observation (Lippuner, *et. al.*, 2009). In this study, real time imaging was used to follow up Rab5 (key factor in phagosome maturation belonging to several groups of Rab GTPase), and the results showed that Rab5 was detectable for short time (two minutes) in the parasite phagosomes, compared to its expression in phagosomes of macrophages treated with latex beads.

The acidic condition inside the phagosomes is essential for the pathogen's degradation, and the *Leishmania* parasite could inhibit acidic condition formation. Vinet, *et. al.*, (2009) have demonstrated that *L. donovani* LPG could interfere with the lipid microdomains of the phagosome membrane which contain gangliosid GMI, which consequently leads to the inhibition of phagosome acidification. Moreover, the *Leishmania* parasite could arrest phagosome maturation at different stages according to the type of infected cells. For example, in bone marrow DCs, the phagosome parasite compartments have been shown to be arrested at the late endosome stage, which might allow the parasite to travel to systemic tissue. In contrast, the phagosome parasite compartment in matured bone marrow DCs requires some lysosome fusion (Kaye and Scott, 2011).

Another interesting reported observation related to *Leishmania* parasite infection is that some species could enlarge the size of the phagosome parasite compartment, and this might support intracellular survival by diluting the acidification effect (Sacks and Sher, 2002). A lysosomal trafficking regulator (LYST) gene regulates lysosome size in cells (Kaye and Scott, 2011). It has been demonstrated that the LYST gene was induced

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following infection of mice macrophages and fibroblasts with *L. amazonensis* amastigotes, and this resulted in big PVs, which increase their survival and proliferation (Wilson, *et. al.*, 2008).

1.4.1.8 Modulation of host cell signalling in *Leishmania* parasite infection

As the *Leishmania* parasite has two different stages during its life cycle which take place in different (invertebrate and vertebrate) hosts, this may give the parasite additional immune evasion mechanisms. An early and rapid modification of host cell signalling pathways by the parasite is required firstly for safe internalization, and secondly for surviving and proliferating inside the host cell.

1.4.1.8.1 Modulation of PKC protein via *Leishmania* parasite infection

Cell biology and signalling pathways are organized by protein phosphorylation, which is controlled by protein phosphatases and protein kinases (Hunter, *et. al.*, 1995; Seger, *et al.*, 1995). The protein kinases C (PKC) family have an important role in macrophage function by regulating the secretions of cytokines such as IFN- γ and TNF- α , both of which are mediators for NO production and the oxidative burst (Shio, *et. al.*, 2012). *Leishmania* parasites could therefore subvert various cell signalling pathways *via* activation of the molecules that play a negative role in cell signalling pathways, or inhibit the molecules, which have a positive function in cell signalling pathways, thereby favouring their survival and disease progression (Shio, *et. al.*, 2012). It has been reported that the ability of *Leishmania* parasites to modulate the PKC activity was related to the presence LPG molecules, which could occupy the binding site of PKC active domain (McNeely and Turco 1987; McNeely, *et. al.*, 1989, Descoteaux, *et. al.*, 1992; Descoteaux and Turco, 1999).

Myristoylated alanine-rich C-kinase substrate (MARCKS), one of the important PKC substrates binds to plasma membrane and has many functional roles in different cell types, including macrophages (Olivier, *et. al.*, 2012). It has been reported that MARCKS is up regulated following macrophage treatment with LPS, and this is facilitated by cytokine secretion (Corradin, *et. al.*, 1999). The same group showed that MARCKS activation was inhibited in macrophages infected with *L. major*. Moreover, the role of GP63 in this process has also been illustrated using GP63 inhibitors.

Another mechanism which contributes to intracellular parasite clearance and the controlled by PKC activity is the generation of Reactive Oxygen Intermediates (ROI) (Murray, 1982). The unique role of ROI in parasite control was demonstrated by using ROI-deficient mice, which showed susceptibility to *L. donovani* infection. However, these mice eventually controlled the infection (Murray and Nathan, 1999).

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Olivier, *et. al.*, (2005), have reported that the production of ROI from human monocytes is altered after infection with *L. donovani* promastigotes as result of PKC activation.

It is noteworthy that the subversion of PKC may be *Leishmania* spp dependent, since Corradin, *et. al.*, (1999) have reported the responsibility of GP63 protein in *L. major* infection with respect to this process, whilst Shadab and Ali, (2011) have described the importance of LPG protein in *L. donovani* infection. In addition, Olivier, *et. al.*, (2005) reported that LPG from *L. donovani* amastigotes was also able to modulate PKC activity. These findings suggest other mechanisms via which promastigotes and amastigote-derived molecules can modulate signalling pathways in infected cell. The consequences of PKC modulation following the parasite infection are summarized in the following diagram (Figure 1.4.1).

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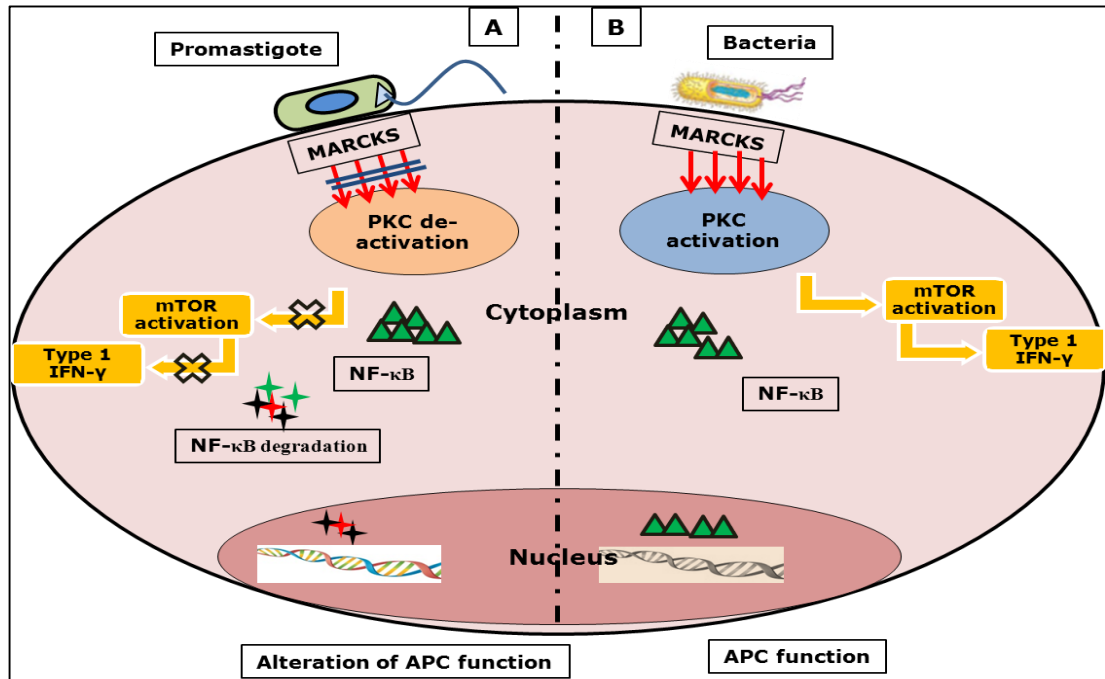


Figure 2.2-1 Diagram illustrating the modulation of host cell signalling

Following the infection, membrane or secreted form *Leishmania* parasite GP63 can block and inhibit an important PKC substrate MARCKS (A), this subsequently induces high destruction of host cell functions, and also the de-activation of mTOR protein kinase, an important translation to initiate IFN- γ response, and arrests the NF- κ B in the cytoplasm. In contrast, MARCKS is strongly up regulated after stimulation of macrophages with LPS (B), and this leads to a type 1 immune response (Modified from Olivier, *et. al.*, 2012)

1.4.1.8.2 JAK2/STAT1- and IFN- γ -mediated signalling

One of the most important discoveries in cell biology with regard to activation of IFN-transcription is the JAK2/STAT1 pathway. A group of factors belonging to Janus kinase, also known as Just Another Kinase (JAK), can activate this family. In addition to their reported role in cell proliferation, migration, differentiation and apoptosis, JAK signalling pathways have a significant role in the immune response. Moreover, JAK protein pathways are triggered as a response to cytokines or growth factors binding to the host cells (Woldman, *et. al.*, 2001). The STAT family, including STAT1, is located in the cell cytoplasm as the inactive form. The extracellular binding of cytokines generates the activation of membrane-bound JAK which subsequently phosphorylates STAT1. This leads to migration of STAT to the nucleus, its binding to specific DNA sequences, and leads to the transcription of specific genes (Olivier, *et. al.*, 2005).

The presence of the virulence molecules that coat the surface of *Leishmania* promastigote surface such as GP63, LPG2, CPB (Shio, *et. al.*, 2012), can dephosphorylate the JAK2/STAT1 signalling pathway, and this directly alters the

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modulation of such IFN- γ , IL-12, iNOS (inducible nitric oxide) and NO (Nitric oxide) in APCs infected with the parasites (Shadab and Ali, 2011).

The importance of STAT1 with regard to parasite clearance was demonstrated by Harnett, *et. al.*, (1999) who showed that STAT1^{-/-} mice developed severe lesions following infection with *L. major* promastigotes. However, infected STAT1^{-/-} mice infected with *L. donovani* exhibited fewer pathological effects compared to control mice (Anstead, *et. al.*, 2001).

Many studies have reported that the *Leishmania* parasite can modulate the mitogen activated protein kinase (MAPKs), a key signalling pathway which mediates responses to extracellular stimuli and regulates APCs function such as the generation of NO and the secretion of proinflammatory cytokines (Seger and Krebs, 1995; Liu and Uzonna, 2012). The effect of *Leishmania* parasites on MAPKs was evaluated on the basis of PTP (Protein tyrosine phosphatases) which was found to be activated as a consequence of *Leishmania* infection (Forget, *et. al.*, 2006).

Attachment of the *Leishmania* parasite to macrophages can cause super activation of a specific family of proteins called PTP (protein tyrosine phosphatases) such as SHP-1, PTP1B and TCPTP, which in turn inhibit and interfere with the JAK kinase family (Shapira and Pinelli, 2006; Silverman, *et. al.*, 2008). Gomez, *et. al.*, (2009), have shown the role of GP63 in activation of PTPs family using wild type *L. major* and GP63 knockout *L. major*. This was further investigated by Hassani, *et. al.*, (2011) who reported that activation of PTPs family was due to the secretion of GP63 and CPB by macrophages infected with *L. mexicana*.

A study by Abu-Dayyeh, *et. al.*, (2010), has revealed the effects in life cycle phases of *L. mexicana* on the activation of PTPs. This group showed that both stages of *L. mexicana* (promastigotes, amastigotes) were able to activate the SHP-1 subunit, whereas the PTP1B subunit was activated in macrophages infected with promastigotes, but not with amastigotes. In addition, *L. mexicana* knockout CPB (Lmcpb^{-/-}) failed to activate PTP1B compared to wild type promastigotes, and this may indicate the role of CPB in the activation of PTPs in *L. mexicana* infection. Activation of PTPs during *Leishmania* infection also has an indirect effect on down regulating the production of IFN- γ . Abu-Dayyeh, *et. al.*, (2008), have reported that SHP-1 can bind and block the IRAK-1, an important kinase substrate for TLRs signalling. This binding subverts the macrophage innate immune response, which is triggered by production of TNF- α , IL-

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12 and NO. Activation of PTPs following the parasite infection is summarized in (Figure 1.4-2). Nevertheless, *Leishmania* species can selectively trigger the production of some pro-inflammatory and inflammatory cytokines, chemokines at different time points during infection, which attract more host cells to the site of infection.

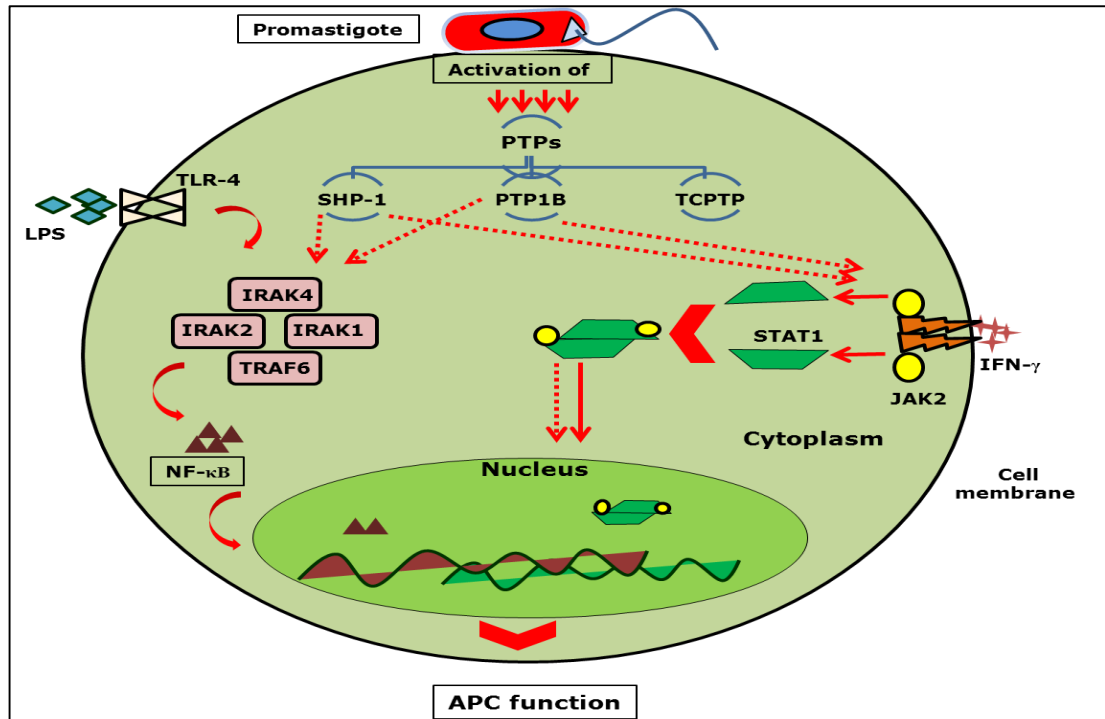


Figure 2.2-2 Diagram illustrating the activation of PTPs following the infection

PTP proteins (SHP-1, PTP1B and TCPTP), are usually up regulated following *Leishmania* parasite infection. Super activation of this family has a negative effect on JAK2/STAT1 cell signalling pathway. The SHP-1 can react with JAK2 kinase and prevent STAT1 phosphorylation and its translocation to the nucleus, this suppresses NO production. PTPs family can also interfere with the activation of TLRs *via* effects on their substrate family, which belongs to PKC such as IRAK1, IRAK2 and IRAK3 (Modified from Olivier, *et. al.*, 2012).

1.4.2 Adaptive immune response to *Leishmania* parasite infection

The failure of an innate immune response to *Leishmania* parasite infection may enable parasite entry into a wide range of immune cells. This can also lead to a correct signalling for the initiation of the adaptive immune response. Generally, a strong immune response leads to the activation and proliferation of required T cells which can stop the infection and end with memory immunity for future infections. The mechanisms of adaptive immune response are modulated in *Leishmania* infection.

Infectious diseases that are caused by parasitic infection such as Leishmaniasis are characterised by the lack of adequate T cell response. However, studies using different strains of mice that are susceptible (Balb/c) and resistant (C57LB/6) to disease have identified the roles of the adaptive immunity in *Leishmania* spp infection.

T lymphocytes (T cells) only recognize the antigens presented on the surface of APCs via MHC class I or MHC class II molecules. The two subsets of the T cells population: cytotoxic T cells, which identified by expression of CD8, and helper T cells, which express CD4 molecules, activated according to the type of MHC molecules, located on the surface of the APC. If the antigens were loaded into MHC class I molecules, CD8 would be activated to kill the infected cells, while, CD4 cells would be activated as consequence of antigen presentation via MHC class II molecules. These cells subdivided into two functional groups: Th1 and Th2 T cells. In spite of its failure to control the *Leishmania* parasite infection, innate immunity has a pivotal role in activation of the most effective anti-parasite (intracellular) immune response. The three main steps required to increase the number of specific T cells are: antigen processing and presentation, co-stimulatory molecules and production of the related cytokines (Kaye and Scott, 2011), and they all start in innate immune compartments. It has been reported that all T cell sub populations take part in *Leishmania* parasite infection, and the role of each subset varies according to the type APCs involved and the *Leishmania* spp, which in turn subvert them toward their advantage.

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1.4.2.1 Cytotoxic T cells and *Leishmania* parasite infection

Generally, the response of CD4⁺ Th1 cells has the major role in *Leishmania* infection by controlling the secretion of IFN- γ . This leads to activation of other macrophages and induces DC maturation as well as the activation of CD8⁺ cells (Kaye and Scott, 2011). Although the role of CD8⁺ cells in immunity to intracellular parasites such as *Toxoplasma gondii* and *T. cruzi*, and bacterial infections such as *Mycobacterium tuberculosis* has been described, the role of CD8⁺ cells in *Leishmania* infection is still not clearly identified. In addition, the published data are still controversial and there is confusion in many cases. Some of the results illustrate that the role of CD8⁺ cells in parasite killing is species-dependant. For example, the role of CD8⁺ T cells in VL control has been demonstrated in an early study by (Stern, *et. al.*, 1988). In contrast, other studies have shown that the CD8⁺ response is not important in the control of some *Leishmania* species causing CL, such as *L. major* (Muller, *et. al.*, 1993). Another study has reported that these cells were required to control disease progression when resistant C57BL/6 mice were infected with a low dose of the *L. major* parasite (Belkaid, *et. al.*, 2002). Results of Faria, *et. al.*, (2009) demonstrated that CD8⁺ T cells are associated with lesion progression in patients with CL caused by *L. braziliensis* infection. This later finding was not in agreement with the findings of Belkaid, *et. al.*, (2002) who reported that CD8^{-/-} C57BL/6 mice failed to control the *L. major* infection. Recently, more attention has been paid to CD4 cell immune responses and the paradoxical role of the Th1 and Th2 paradigm following exposure to *Leishmania* spp. This might be a promising line of investigation for better undersigning and protective immunity to Leishmaniasis, as well as for the development of effective vaccine candidates.

1.4.2.2 Helper T cells and *Leishmania* parasite infection

Differentiation of Th1 and Th2 immune responses is mostly related to the type of cytokines and its concentration in the plasma after infection (Maggi, *et. al.*, 1992). If Th0 cells are stimulated by IFN- γ and IL-12, then they differentiate into Th1, whereas stimulation with IL-4 and IL-10 leads to Th2 differentiation (Powie, *et. al.*, 1993).

1.4.2.2.1 Th1 response

Many studies have reported that resistance to *Leishmania* parasite infection is related to the induction of Th1 immune responses. In human Leishmaniasis, there is a

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significant association between the type 1 immune response and resistance to cutaneous Leishmaniasis, and this associated with a high production of IFN- γ (Romao, *et. al.*, 2009). On the other hand, mucosal lesions and chronic CL are characterised by a mixture of Th1 and Th2 cytokines (Mendez, *et. al.*, 2001). Animal models such as susceptible and resistant mice strains have had a great impact on current knowledge and understanding of the balance between Th1 and Th2 paradigms after *Leishmania* parasite infection. In susceptible Balb/c mice and resistant C57BL/6 infected with *L. major* promastigotes, the IL-4 driven Th2 response is associated with disease progression in Balb/c mice, whereas IL-12 driven Th1 responses are associated with parasite resistance in C57BL/6 mice. However, the variation between Th1 and Th2 responses across infections with different *Leishmania* spp remained unclear.

The role of Th1 or Th2 immune response has also been investigated using IL-12^{-/-} C57BL/6 mice. These mice are unable to control the parasite infection, which was associated with low levels of IFN- γ mRNA expression and high levels of IL-4 mRNA expression (Hondowicz, *et. al.*, 1997). Additional experiments have shown that treatment of resistant C57BL/6 mice with neutralizing anti-IL-12 antibodies during the course of *Leishmania* infection results in disease progression. Many studies have reported the role of IL-10 in the progression of *Leishmania* parasite infection (Belkaid, *et. al.*, 2001), have illustrated that depletion of IL-10 in mice infected with *L. major* promastigotes controls parasite infection. Recent studies using enzyme-linked immunosorbent assay (ELISA) to study cytokine profiles in the sera of patients infected with Leishmaniasis have found that levels of TNF- α , IL-12, IL-4, and IL-10 are increased in patients with active lesions, thereby suggesting a role of these cytokines in *Leishmania* infection (Rodriguez, *et. al.*, 2007).

Other studies have demonstrated that activation of the two subsets of Th helper lymphocytes by the administration of adjuvant mixed with GP63 isolated from *L. donovani* promastigotes results in the prevention of VL symptoms in susceptible Balb/c mice (Nathan, *et. al.*, 1983; Bhowmick *et. al.*, 2008). Therefore, the correlation between Th1 response and *Leishmania* parasite infection control seems to be species-dependent.

1.4.2.2.2 Th2 response

The failure of an early and adequate Th1 immune response after *Leishmania* infection is responsible for Leishmaniasis development. A considerable number of studies have

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shown that progressive lesions are associated with a high Th2 immune response (Bourreau, *et. al.*, 2003; Murray, *et. al.*, 2005).

For example, susceptible Balb/c mice infected with *L. major* and treated with anti-IL-4 monoclonal antibodies can successfully overcome their infection (Chtelain, *et. al.*, 1992). Other studies have shown that the high levels of IL-4 cytokine after *Leishmania* parasite infection is related to the high number of CD4⁺ T cells. These cells were characterized by the expression of V β 4/V α 8 T cell receptors which successfully bind to the *Leishmania* antigen LACK (*Leishmania* homologue of receptor for activated C kinase) (Launois, 1995; Melby, *et. al.*, 2001). Donnelly, *et. al.*, (2005) reported the role of LACK antigens in the stimulation of Th2 immune responses and their role in developing progressive lesions in susceptible Balb/c mice has been fully verified. Results have also shown that infected Balb/c mice with LACK knockout *L. major* failed to activate targeted transgenic T cells with V β 4/V α 8 receptor. However, the LACK antigen itself is highly immunogenic and had been widely considered as a vaccine candidate for Leishmaniasis. Melby, *et. al.*, (2001) investigated the capacity of LACK to influence the development of Leishmaniasis, and showed that immunised Balb/c mice that had been immunised with recombinant LACK antigen were protected from *L. major* infection. The protection was a consequence of the shifting of the immune response from Th2-IL-4 towards Th1-IFN- γ dependent. In contrast, in the case of challenging with *L. donovani*, LACK vaccine did not protect against VL, even though the immune response was the same as in the case of infection with *L. major*. However, these findings emphasis the variation of immune response to different species of *Leishmania* parasite. Therefore, an effective immune response to *Leishmania* parasite infection is dependent on the activation of both innate and adaptive immunity, as shown in (Figure 1.4-3).

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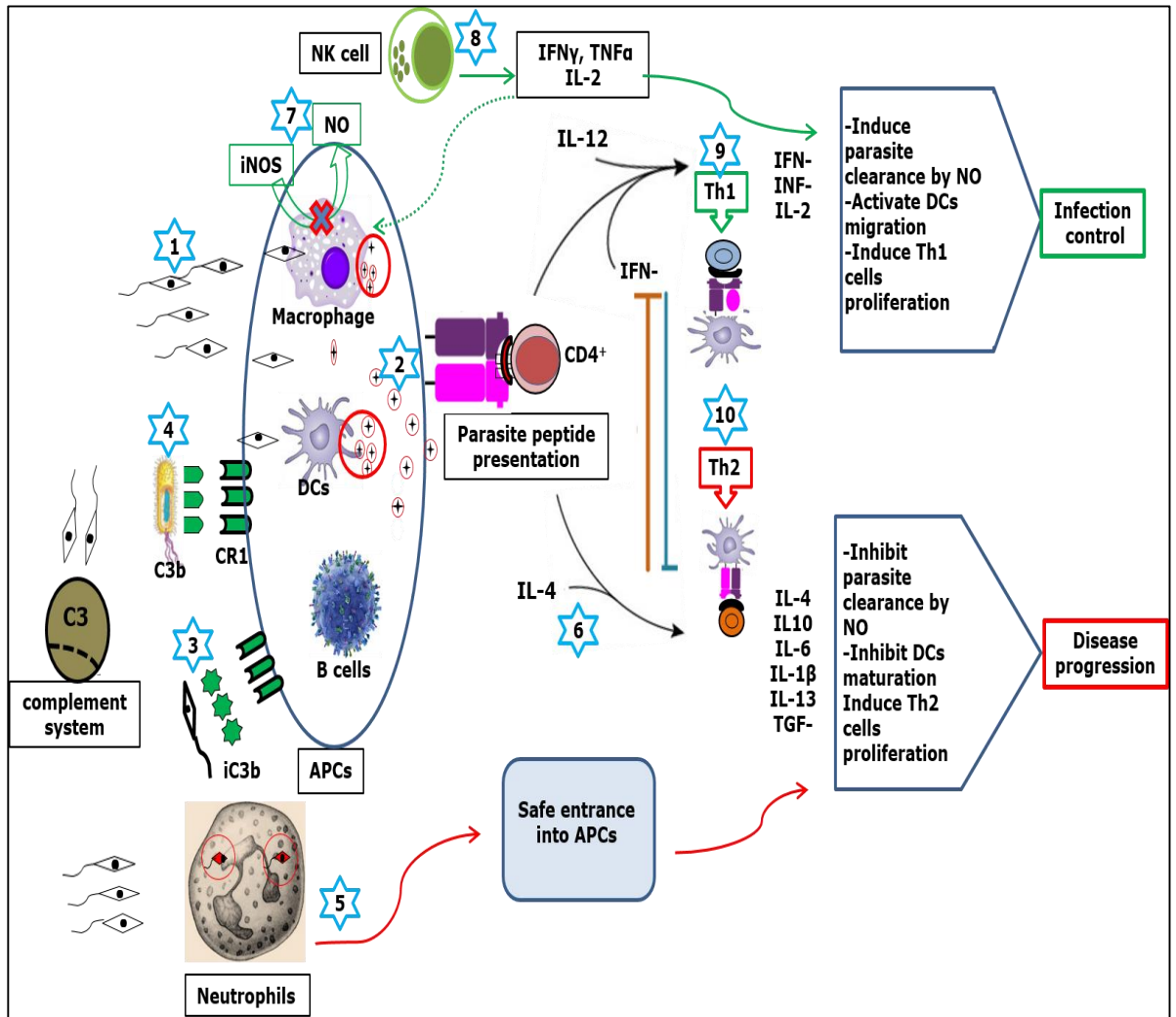


Figure 2.2-3 Interactions between *Leishmania* parasite and host immune cells

The graph shows there is no distinguish line between the innate adaptive and immune response, since the parasite can either interact with various cell types and modulate their cytokines production. For example, *Leishmania* promastigotes (1) transform into the amastigote stage inside infected cells (2), and they can fix complement C3b subunit into iC3b (3), which prevents them from opsonisation compared to the LPS (4). Promastigotes can also use neutrophils for safe entrance into macrophages (5). They are able to activate the migration of DCs to the site of the infection and down regulate their cytokines towards a Th1 immune response, which allows them to travel away to the systematic tissues (6). Most importantly they can inhibit iNOS which negatively downstream NO secretion (7). NK cells also will be activated following *Leishmania* infection, and secrete IFN- γ (8). Activation of adaptive immune through Th1 (9) or Th2 (10) Leads to parasite clearance or disease progression respectively.

1.5 *Leishmania* vaccines

Despite all the comprehensive attempts to date, a good vaccine model has not been developed. Currently, treatment for Leishmaniasis is chemotherapy dependent, such as Amphotericin B and Pentamidine, which are compromised by unpleasant side effects and the high cost. Therefore, working towards vaccination may be more appropriate in comparison to chemotherapy treatment. A good anti-*Leishmania* vaccine would have several characteristics such as safety and stability, availability to the people in need, induction of good CD4 and CD8 T cells responses and be effective for both CL and VL. Different methods of vaccination have been used to control *Leishmania* infection, and this have given a promising result in experimental models. Although these vaccines failed to provide protection, they significantly increased the current knowledge of the complexity of immune response to the *Leishmania* parasite. The first generation of *Leishmania* vaccines, which include Leishmanisation and killed vaccine, and second generation which include genetically modified parasites, are briefly described below.

1.5.1 Leishmanisation and killed vaccine

Leishmanisation is a process whereby live virulent parasite is introduced to the host in order to produce a long lasting immunity which is associated with activation of T cells mediated immunity and protection against re-infection with the parasite (Nadim, *et. al.*, 1983). This method was widely used in the 1970s and 1980s in endemic areas such as Iran and the Soviet Union (Greenblatt, 1988; Khamesipour, *et. al.*, 2005). WHO later banned this method in many countries, because of safety issues (some vaccinated patients developed non-healed lesions). Afterwards, the focus of vaccine development was shifted towards using a killed preparation of the parasite. This was introduced to the world by Brazilian scientists in the late 1930s, and this type of vaccination was performed by the inoculation of the host with killed promastigotes. Although, this method of vaccination is safer compared to Leishmanisation, this method was compromised by the low effectiveness of protection in endemic areas (Mayrink, *et. al.*, 1985). For example, a vaccine called Leishvacin derived from *L. amazonensis* (Marzochi, *et. al.*, 1998), was used in Ecuador and Colombia and was safe, but not effective (Velez, *et. al.*, 2005). In addition, the observation that vaccinated individuals developed Th1 immune response without controlling the infection, which also was in agreement with others (Gicheru, *et. al.*, 2001), suggests that the Th1 immune response was less important in controlling CL. However, a good protection was achieved using

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killed *L. major* against *L. donovani* infection in canine and hamster models (Mohebbi, *et. al.*, 2004).

1.5.2 Live-attenuated vaccines

In this method, vaccination is performed with non-pathogenic parasites, which can be taken up by different host cells and stimulates a long-term immune response. This method was more effective in controlling parasite infection compared with Leishmanisation and killed vaccination methods. Attenuated parasites can be produced by various methods, such as long term culturing under gentamicin pressure, and knocking out some genes (Kedzierski, *et. al.*, 2006), and different results have obtained as a consequence. For example, *L. major* promastigote knockout dihydrofolate reductase-thymidylate synthetase (DHFR-TS) gene (required for a long term survival in the host), induces some protection in murine models infected with *L. major* and *L. amazonensis*, but failed to produce any protection against infection with *L. major* and *L. amazonensis* in infected primate models (Amaral, *et. al.*, 2002). Although the attenuated parasite might be produced by a knockout virulence gene such as LPG1 gene (encoding a putative galactofuranosyl transferase involved in the biosynthesis of the virulence lipophosphoglycan) and cysteine proteinase, these parasites could still induce the infection (Huang and Turco, 1993). A study by Uzonna, *et. al.*, (2004) showed that *L. major* knockout LPG2 gene (responsible for transportation of GDP mannose to Golgi apparatus) gave Balb/c mice protection from homologous infection, and that the protection induced by attenuated parasites was not related to the Th1 immune response, as was indicated by IFN- γ production. However, a study by Carrió, *et. al.*, (2011) demonstrated that immunisation of Balb/c mice with of *L. infantum* HSP70-II knockout gene (encoding heat-shock response), induced a high level of protection against *L. major* infection. The protection was associated with high levels of NO concentration and Th1 immune response, as determined on the basis of an ELISA analysis of anti-*Leishmania* IgG antibody subclass.

1.5.3 Recombinant protein vaccines

Recombinant protein vaccine consists of protein fragments of the pathogenic organism that have been produced in cells engineered genetically to express particular proteins, for example, bacteria and yeast. These antigens (synthesised proteins) can be successfully used as a vaccine model (Khamesipour, *et. al.*, 2006). Due to their high immunogenicity, the immune system will raise specific required antibodies against the

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introduced proteins, which eventually protect the host from the infection. This method was widely used to control many diseases including *Leishmania* infection. In almost all known *Leishmania* spp, GP63 protein was considered to be highly immunogenic, and was therefore used as a target for vaccines against Leishmaniasis. For example, use of GP63 as a recombinant vaccine induced some protection in Vervet monkeys (*Cercopithecus aethiops*) challenged with *L. major* (Olobo, *et. al.*, 1995). Other important immunogenic proteins used as recombinant vaccines are cysteine proteinases. Rafati, *et. al.*, (2002), reported that recombinant CPB and CPA proteins mixed with poloxamer 407 as an adjuvant induced the immune response in Balb/c mice infected with *L. major*. Although recombinant CPB protein induced partial protection, recombinant CPA did not. In addition, the protection of infected Balb/c mice was associated with CD8 T cells, since depletion of these lymphocytes using depleting CD8 antibodies in mice immunised with recombinant CPB led to disease progression. In this type of vaccine, a single protein is usually used, however, some studies have demonstrated that using more than one immunogenic protein at once induces a stronger degree of protection. For example, recombinant LmSTI1 and TSA protein gives good protection in Balb/c mice (immunised three times) challenged with freshly isolated *L. major* amastigotes, compared to recombinant TSA mixed with IL-12, which induces partial protection. However, immunisation with a mixture of both proteins induced strong protection in a murine challenged model (Campos-Neto, *et. al.*, 2001).

1.5.4 DNA vaccine

DNA vaccines are based on introducing the genetic material from the pathogen of interest to the mammalian host, instead of introducing the antigen itself (Liu, *et. al.*, 2005; Choi, *et. al.*, 2009). Although traditional vaccines have successfully protected humans from some diseases, they failed to protect against many other diseases. In the last decade, genetic vaccines have been extensively investigated as promising future candidates to prevent the spread of many infectious diseases. Unlike the majority of traditional vaccines, DNA vaccines are becoming increasingly more reliable due to their unique features. Since they do not introduce any live pathogenic element to the immunised subject, they are considered to be safer (Sheets, *et. al.*, 2006). In addition, plasmid DNA is more stable and relatively easy to prepare compared to others. This makes them affordable to people in developing countries (Kedzierski, *et. al.*, 2006; Al-Wabel, *et. al.*, 2007). It has been reported that DNA vaccines are highly immunogenic, so that they are able to stimulate both humoral and cellular immune responses against encoding proteins (Liu, *et. al.*, 2005; Ganges, *et. al.*, 2008).

Following immunisation, plasmid DNA is taken up by host cells, and the proteins of interest synthesised. These then stimulate either a cellular or antibody mediated immune response. Once the inserted gene is translated into protein, it may follow an MHC class I or a MHC class II pathways or both. If the encoding protein is digested inside the cell cytoplasm due to proteasome enzymes, this leads to peptide circulation and loading onto newly sensitized MHC I molecules in the endoplasmic reticulum in the presence of other supportive components. MHC class I peptide complexes are then transported through the Golgi complex to the cell surface (endogenous pathway). The presentation of loading peptides on the APC surface by MHC class I stimulates cytotoxic T lymphocytes (CD8⁺), and induces a cell-mediated immune response (Anderson and Schneider, 2007; Choi, *et. al.*, 2009).

Alternatively, the proteins encoded by DNA vaccines can be released outside the transfected cells, and taken up by APCs as foreign antigens. These proteins will be digested into peptides inside the endosomes, treated as extracellular antigens and loaded on MHC class II molecules, to be transported into the cell surface by the Golgi complex (exogenesis pathway). Peptide presentation by MHC class II stimulates helper T lymphocytes (CD4⁺), which facilitate the generation of antibodies by B cells (Liu and Ulmer, 2005). Moreover, plasmids derived from the bacteria are highly immunogenic,

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as the backbone contains un-methylated cytosine-phosphate-guanosine motifs (CpG) which stimulates the innate immune response through TLR9 (Anderson and Schneider, 2007; Rottembourg, *et. al.*, 2010). The different scenarios of immune responses following DNA immunisation are summarised in (Figure 1.5-1).

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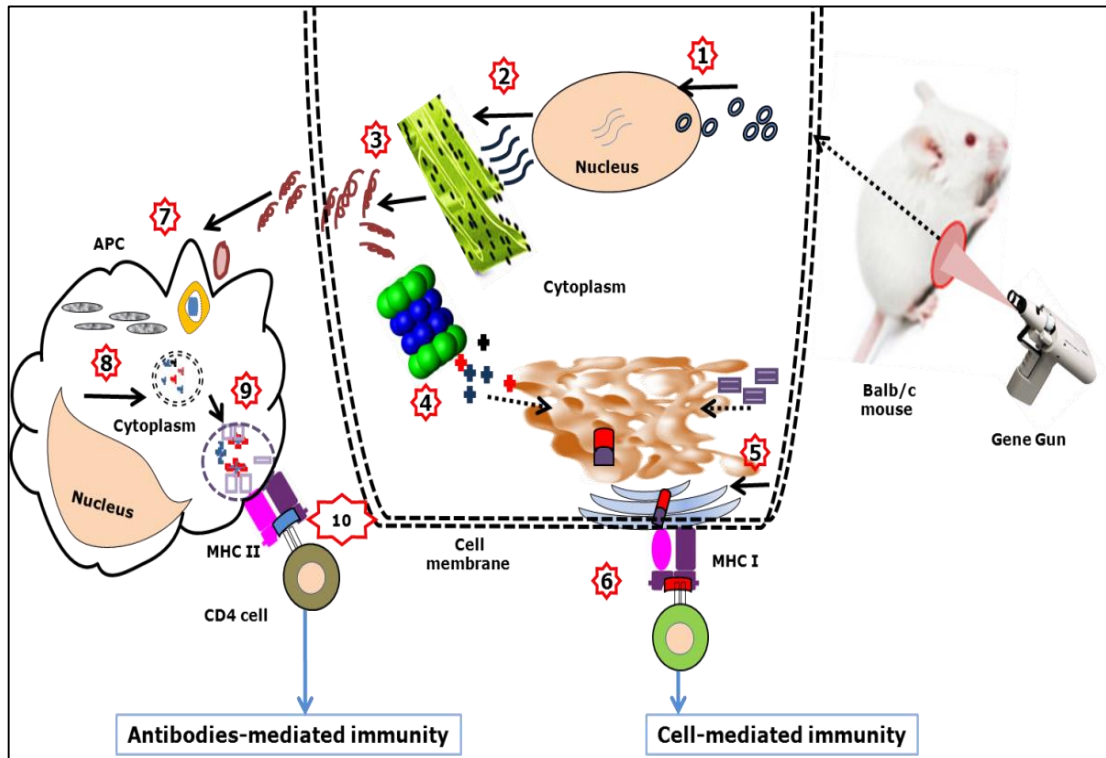


Figure 2.2-1 Facts related to DNA vaccines

Gene gun immunisation with plasmid encoding a gene of interest will be taken up by cells and translated into mRNA: (1). This travels to the cytoplasmic rough reticulum to produce the protein (2). In the Endogenous Pathway, the encoding protein (3) is digested inside the proteasome to peptides (4). These enter the smooth endoplasmic reticulum together with newly generated MHC class I. Peptide-MHC class I complex travels through the Golgi complex to the cell surface (5). The complex can be recognized by CD8 (6). In the exogenous pathway, the encoding protein is secreted outside the cell and taken up by APCs (7). Endosome-lysosome fusion takes place (8). The protein is digested into peptides, which are loaded onto MHC class II in specific small compartments (9). This process ends by traveling to APC surface and the CD4 subset is stimulated (10). (Modified from Schirmbeck and Reimann, 2001).

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DNA vaccines, encoding one or more *Leishmania* genes, have been used experimentally to protect animal models from developing lesions when they are challenged with live *Leishmania* parasites. Al-Wabel, *et. al.*, (2007) have reported that immunisation of susceptible Balb/c mice with recombinant nucleoside hydrolase protected against *L. major* infection, and that the protection of immunised mice was related to enhancement of a Th1 response. In a similar study, immunisation of C57BL/6 mice with CpG DNA along with live *L. major* strongly stimulates dermal, but not bone marrow DCs to produce IL-2 and IFN- γ (Laabs, *et. al.*, 2009).

Hence, DNA vaccines are a promising approaches for preventing or eliminating widespread *Leishmania* infection in both developing and developed countries.

The current project therefore, was designed to test and compare the immunogenicity of three different *Leishmania* genes cloned into three different plasmids:

- i. *Leishmania mexicana* GP63.
- ii. *Leishmania donovani* centrin1.
- iii. *Leishmania donovani* centrin3.

Each gene will be sub-cloned in three plasmids, which are VR1012, pcRT7/CT-TOPO and pcDNA3.1/Higro(-).

1.5.4.1 *Leishmania* parasite GP63 protein

The biological roles of GP63 as virulence factor have been previously addressed (section 1.3.3) its role as vaccine candidate will be discussed in this section. The surface of most species that belong to the Trypanosomatidae family is coated with 63-kDa GPI-anchored protein known as GP63, which is present at 5×10^5 molecules per parasite (Etges, *et. al.*, 1986). In *Leishmania* species that have been studied, at least one or two isoforms of the GP63 gene are presented in both stages of the *Leishmania* parasite life cycle: the extracellular metacyclic promastigotes and intracellular amastigotes (Ilg, *et. al.*, 1993; Inverso, *et. al.*, 1993).

Since GP63 is presented in all *Leishmania* spp, it has been a vaccine target for many researchers (Handman, *et. al.*, 1995). GP63 was used as a recombinant protein vaccine, which failed to protect immunised mice from infection with *L. major*, but it did give partial protection in immunised monkeys challenged with *L. major* (Olobo, *et. al.*, 1995). The GP63 gene was used in combination with other genes as a DNA vaccine. For example, the multi-antigenic plasmid DNA vaccine encoding KMP11 (kinetoplastid membrane protein-11), TRYP (Tryparedoxin peroxidase (TRYP), or

1) General introduction

thiol-specific antioxidant (TSA), LACK and GP63 *L. infantum* antigens failed to protect immunised dogs that were challenged with *L. infantum* promastigotes, after four dose of immunisation (Rodríguez-Cortés, *et. al.*, 2007). Interestingly, many have identified the effectiveness of some isoforms of GP63 as a vaccine to *Leishmania* infection, while other isoforms could lead to the exacerbation of Leishmaniasis symptoms (Tsagozis, *et. al.*, 2004).

L. major GP63 has been broadly used as a DNA vaccine, and it has been reported that Th1 immune response Balb/c mice could be enhanced by GP63 DNA immunisation, and that this to a significant reduction in lesions size and the number of parasite burdens (Walker, 1998). On the other hand, immunisation of susceptible Balb/c mice with different isoforms of GP63 cloned into VR1012 (VR1012-GP46, VR1012-GP63 and VR1012-CPb) as DNA vaccine, gives a partial protection from infection with *L. mexicana* (Dumonteil, *et. al.*, 2003).

1.5.4.2 *Leishmania* parasite centrins

Many studies have reported that Centrins, as calcium binding proteins, have fundamental roles associated with microtubule organising centres (CMTOC), (Wolfrum and Salishbury, 1998; Klink and Wolniak, 2001). Three centrin isoforms which vary in terms of characterisation and function are found across many species:

- i. Human centrin1 (HsCEN1).
- ii. Human centrin2 (HsCEN2) and *Chlamydomonas reinhardtii* centrin (CrCEN1) are important in centrosome segregation.
- iii. Human centrin3 (HsCEN3) and yeast centrin (CDC31) are essential in centrosome duplication (Selvapandiyan, *et. al.*, 2001).

Since the role of centrin in cell growth and multiplication has been well defined, *Leishmania* parasite centrins have attracted the attention of many parasite immunologists in attempts to develop attenuated *Leishmania* spp that could be promising vaccine candidates. It has been reported that the growth of *Leishmania* parasites in which the centrin gene has been knocked out was highly affected. For example, the growth rate of *L. donovani* promastigotes knockout centrin gene was arrested at the G2/M stage compared to controls, and also that the growth of the amastigote was strongly inhibited in infected macrophages (Selvapandiyan, *et. al.*, 2001; Selvapandiyan *et. al.*, 2004). Centrins have similar roles within the life cycle of *T. brucei* (Selvapandiyan, *et. al.*, 2007).

1) General introduction

The efficiency of attenuated *Leishmania* parasites as a vaccine has been tested using centrin knockout *Leishmania*. Challenging susceptible Balb/c mice with *L. donovani* LdCEN^{-/-} induced Th1 immune response, compared to mice challenged with normal *L. donovani* (Selvapandiyan, *et. al.*, 2004). The Th1 immune response was correlated with a high production of interferon gamma (IFN- γ), increased IgG2 immunoglobulins as well as production of NO in macrophages. Moreover, immunisation using LdCEN^{-/-} protects against *L. braziliensis* induced MCL (Selvapandiyan, *et. al.*, 2009).

1.5.4.3 VR1012, pcDNA3.1/Hygro(-) and pcRT7/CT-TOPO plasmids

DNA plasmids incorporating the human cytomegalovirus promoter (CMV) such as VR1012 and pcDNA3.1 plasmids generate better gene expression following transfection compared to plasmids with other viral promoters, such as the SV40 promoter (Ali, *et. al.*, 2009). It is well known that VR1012 and pcDNA3.1/Hygro(-) vectors are more effective mammalian plasmids with highly successful rates in DNA vaccination methods (Guo, *et. al.*, 2000). pcRT7/CT-TOPO plasmid is widely known as a prokaryotic vector, but it has been shown to transfect and express genes of interest in mammalian cells (Asteal, 2011). These plasmids were used to develop a DNA vaccine candidate against *Leishmania* parasite infections. For instance pcRT7/CT-TOPO encoding *L. donovani* centrin3 gene provided better protection of susceptible Balb/c mice comparing to pcDNA3.1/Hygro(-) plasmid encoding the same gene (*L. donovani* centrin3), even the empty pcRT7/CT-TOPO gave some protection of Balb/c mice against challenge with *L. mexicana* promastigotes (Asteal, 2011).

1.6 Aims of the study

Vaccination is the method of choice in controlling infectious diseases and since no effective vaccine is available against Leishmaniasis, this project was partly designed to test and compare the immunogenicity of three *Leishmania* genes (*L. mexicana* GP63, *L. donovani* centrin1 and *L. donovani* centrin3) cloned in 3 different plasmids (pcRT7/CT-TOPO, VR1012, and pcDNA3.1/Hygro(-)). The immunogenicity of newly prepared DNA vaccines will be evaluated *in vivo* by a gene gun immunisation method in susceptible Balb/c mice. Immune responses will be analysed in spleen, lymph nodes, blood and bone marrow of immunised and control mice using the following immune assays:

- I) Elispot (Enzyme-linked immunospot) assay to measure the CTL activity in the immunised mice.
- II) ELISA (Enzyme-linked immunosorbent assay) to measure levels of IL-2, IL-4 and IFN- γ in the sera of cultured cells from spleens and lymph nodes stimulated *in vitro* with *L. mexicana* antigens, and to determine total IgG, IgG1 and IgG2a in blood samples
- III) Flow cytometry to estimate the number of T helper cells (CD4) and cytotoxic T cells (CD8) in spleens and lymph nodes.
- IV) Proliferation responses of splenocytes and lymphocytes as a result of *in vitro* stimulation with *L. mexicana* antigens.
- V) A novel *Leishmania* survival assay will be used for the first time in this study to measure the resistance of bone marrow derived macrophages from immunised Balb/c mice to *L. mexicana* infection.

The second aim of this project is to study and evaluate *in vivo* and *in vitro* the effect of long period culturing on *L. mexicana* promastigote infectivity (a model developed in this study).

The third aim of this project is to investigate the interactions between the virulent and avirulent *L. mexicana* promastigotes and susceptible human (U937 monocyte, MonoMac-6 monocytes, and U937 macrophages) as host cells. This will include:

Firstly, study of histocompatibility complex class I (MHC I) expression in target cells.

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Secondly, studying the modulation of gene expression of some Toll- Like Receptors (TLR-1, TLR-2, TLR-4 and TLR-9), Cytokines (IL-1, IL-6, IL-10, IL-12, TNF- α , and TGF- β) and Chemokines (CCL-1, CCL-2, CCL-3, CCL-4, CCL-5 and CCL-22), in target cells following infection with virulent and avirulent *L. mexicana* promastigotes or their antigens measured by qPCR.

Chapter 2/ Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Molecular biology reagents and kits

No.	Product Name	Company	Cat. No.
1	100bp DNA Ladder	Genecraft	GC-015-004
2	1kp DNA Ladder	Promega	G571A
3	5X colourless Go tag R Reaction Buffer	Promega	M7921
4	Alkaline Phosphatase 10X Reaction Buffer	Promega	M133A
5	Alkaline Phosphatase, Calf Intestinal	Promega	M1821
6	Ampicillin, sodium salt	Sigma	A9518
7	AvaI	Promega	R6091
8	BgIII	Promega	R6081
9	Buffer B 10X	Promega	Roo2A
10	Buffer E 10X	Promega	R005A
11	ClaI	Promega	R6551
12	EcoRI	Promega	R6011
13	GelRed TM Nucleic Acid Gel Stain, in Water	Biotium	41003
14	Go Taq R DNA Polymerase	Promega	M3178
15	HaeIII	Promega	R6171
16	Hind III	Promega	R604A
17	iQ SYBR Green Supermix	Bio-Rad	8880
18	InstaGene™ Matrix kit	(Bio-Rad	732-6030
19	KAP A dNTP Mix	Biosystem	KN1008
20	LB Agar High Salt, Granulated	Melford	GL1706
21	LB Broth	Melford	L1704
22	Ligase Buffer, 10x	Promega	C126A
23	MinElute Gel Extraction Kit (50)	Qiagen	28604
24	M-MLV Reverse Transcriptase	Promega	M1701
25	M-MLV RT 5X Buffer	Promega	M531A
26	Nuclease-Free Water	Qiagen	129115
27	Oligo(dT)15 Primer	Promega	C1101
28	Prepaid Barcode labels	MWG	94-050
29	RNase-Free DNase Set (50)	Qiagen	79254
30	RNasin® Ribonuclease Inhibitor	Promega	N2111
31	RNeasy Mini Kit (50)	Qiagen	74104
32	SalI	Promega	R6051
33	Strip Tubes and Caps, 0.1 ml	Qiagen	981103
34	T4 Ligase	Promega	M1801
35	UltraFlux*Flat Cap PCR Tubes	SSI	3220-00
36	Wizard R Plus SV Minpreps	Promega	A1330
37	Xba I	Promega	R6181

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2.1.2 Culture media and supplements

No.	Product Name	Company	Cat. No.
1	Albumin from bovine serum	Sigma	A2153
2	C.T.L Test TM medium	C.T.L	CTL-010
3	DPBS 0.0095M(PO4) w/o Ca and Mg	Lonza	BE12-167F
4	Foetal Bovine Serum	Fisher	SV30143.03
5	Fungizone	Invitrogen	15290-018
6	Geneticin® Selective Antibiotic	Life technologies	10131-019
7	HEPES Buffered Saline Solution	Lonza	CC-5022
8	Kanamycin solution from Streptomyces kanamyceticus	Sigma	K0254
9	L-Glutamine 200mM 100ml	Lonza	BE17-605E
10	Lipofectamine® 2000 Transfection Reagent	Life technologies	11668027
11	Lipopolysaccharides from Escherichia coli	Sigma	L8274
12	Non-essential amino acids (NEAA)	Sigma	M7145
13	OPI Media Supplement	Sigma	O5003
14	Penicillin 5,000, Streptomycin. 5,000 U/ml	Lonza	DE17-603E
15	RPMI 1640 without L-glutamine	Lonza	BE12-167F
16	Schneider's Drosophila Medium, Modified	Lonza	04-351Q
17	Sodium Pyruvate Solution	Lonza	BE13-115E
18	Staphylococcal enterotoxin B (SEB)	Sigma	S4881
19	Trypsin/EDTA Solution 100 ml	Lonza	CC-5012
20	Zeocin Selective Antibiotic	Invitrogen	469270

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2.1.3 Antibodies and kits

No.	Product Name	Company	Cat. No.
1	Anti-human CD14: FITC	Biolegend	325603
2	Anti-human CD68: FITC	Biolegend	333805
3	ELISpot Blue Colour Module	R&D Systems	SEL002
4	FcR Blocking Reagent, human	Miltenyi Biotec	130-059-901
5	F4/80 Antibody - Cl:A3-1	Serotec	MCA497FT
6	Goat anti-mouse IgG2a:FITC	Serotec	STAR133F
7	Goat anti-mouse IgG: HRP	Abcam	Ab6789
8	Goat anti-mouse IgG2a: HRP	Serotec	STAR133P
9	GP63 antibody	Gen Flow	96-126
10	Greiss reagent system	Promega	G2930
11	Hamster anti mouse CD11c: FITC	Serotec	MCA369F
12	InstaGene™ Matrix kit	Bio-Rad	732-6030
13	Mouse anti mouse MHC class I:FITC	Serotec	MCA2189F
14	Mouse IFN gamma DuoSet ELISA Kit	R&D Systems	DY485
15	Mouse IFN- γ ELISpot Development kit	R&D Systems	SEL485
16	Mouse IgG1: FITC κ Isotype ctrl (FC)	Biolegend	400109
17	Mouse IgG2b negative control: FITC	Serotec	MCA691F
18	Mouse IgG2b, κ Isotype Ctrl	Biolegend	400309
19	Mouse IL-12p70 DuoSet ELISA Kit	R&D Systems	DY419
20	Mouse IL-4 DuoSet ELISA Kit	R&D Systems	DY404
21	Protein Kit	Sigma	B9643
22	Rabbit anti mouse IgG1: HRP	Serotec	OBT1508P
23	Rat anti-mouse CD3:RPE-Alexxa Fluor®647	Serotec	MCA500P64
24	Rat anti-mouse CD4: FITC	Serotec	MCA1107F
25	Rat anti-mouse CD8: RPE	Serotec	MCA609PE
26	Rat IgG2a negative control: FITC	Serotec	MCA1212F
27	Rat IgG2a negative control: RPE	Serotec	MCA1212P
28	Rat IgG2a negative control: RPE-Alexxa Fluor®647	Serotec	MCA1212P647

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2.1.4 Chemicals

No.	Product Name	Company	Cat. No.
1	Bovine Serum Albumin	Sigma	A2135
2	2-Mercaptoethanol	Sigma	M3148
3	Calcium chloride	Sigma	499609
4	Dimethyl Sulphoxide (DMSO)	Sigma	D2650
5	Ethanol, absolute	Sigma	E7023
6	Ethanol, anhydrous	Sigma	676829
7	Giemsa Stain, Modified Solution	Sigma	48900
8	Glycerol for molecular biology	Sigma	G5516
9	Glycine	Sigma	G8898
10	Gold Microcarriers	Bio-Rad	165-2263
11	Hemin	Sigma	H9039
12	IsoFlow™ Sheath Fluid	Coulter	72006
13	Isopropanol	Sigma	19516
14	Latex beads	Sigma	L3030
15	MicroScint™ ⁰	Perkin Elmer	6013611
16	Paraformaldehyde	Sigma	158127
17	Phenol:Chloroform:Isoamyl Alcohol	Sigma	P3803
18	Phorbol 12-myristate 13-acetate	Sigma	P1585
19	Polyvinylpyrrolidone (PVP)	Sigma	PVP360
20	Potassium acetate	Sigma	P1190
21	Potassium chloride	Sigma	P9541
22	Potassium hydroxide	Sigma	P5958
23	Potassium phosphate monobasic	Sigma	P5666
24	Sodium azide	Sigma	S2002
25	Sodium hydroxide	Sigma	S5881
26	Spermidine	Sigma	S0266
27	Sulphuric acid (stop solution)	Sigma	339741
28	Thymidine [6- ³ H]	Perkin Elmer	NET3550 01MC
29	Trizma® base, minimum	Sigma	T1503
30	Trizma® hydrochloride	Sigma	T3253
31	Trypan Blue Solution	Sigma	TB154
32	Tween®20	Sigma	P2287

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2.1.5 Equipments

No.	Equipment Name	Company	Cat. No.
1	-20 °C freezer	Les	2C000062
2	4°C fridge	Les	2C000062
3	4°C Mistral 200R centrifuge	MSE	5696/06/27
4	-80°C Freezer	Sanyo	08070722
5	800W compact microwave	Sanyo	SY05/0058
6	Autoclave	Rodwell	00000
7	Bench top vortex mixers	Scientific Industries	50HZ
8	Block heater	Stuart	SBH130D
9	CK2 microscope	Olympus	110605
10	Class II microbiological safety cabinet	Envait	C14299
11	Class II microbiological safety cabinet	walker	C14296
12	CO ₂ water jacketed incubator	Forma Scientific	27555-1063
13	Confocal microscope	Leica	510000141
14	Cooled incubator	LMS	9140/06LR
15	Elispot plate readers	(CTL)	
16	EV243 consort mini power supply	Gene Flow	P5-0040
17	Fluorescence microscope BX51TF	Olympus	6F22612
18	Gallios, 10 colour flow cytometer	Beckman Coulter	1269903
19	Gel Documentation	<i>In Genivs</i>	1032
20	Hotplate with stirrer, aluminium plate analogue	Geneflow	SB162
21	Micro-centrifuge	Heraeus	50/60HZ
22	Microprocessor pH meter	Hanna	500258
23	Multi Sub Midi Horizontal Gel Unit	Geneflow	G9-0010
24	Nanodrop® 8000 8 Sample Spectrophotometer	Labtech	0388
25	qPCR machine	Rcorbett	R070571
26	Scintillation Top Counter	Packard	5069256
27	Shaker incubator	Stuart	R0001040
28	Sonicator	Fisher Scientific	183422050
29	Spectra fluor plate reader	Tecoan	93437
30	Tc-3000G techne gradient thermal cycler	Geneflow	T2-1022
31	Transilluminator	Genetic Research	S/90/090
32	Tubing Prep Station	Bio-Rad	1237
33	Water bath	Grant	OE963900
34	Helios gene gun	Bio-Rad	165-2431
35	Pellet pestles	Sigma	Z359971

2) Materials and Methods

2.1.6 Consumables

No.	Name of product	Company	Cat. No.
1	0.5 ml Micro tubes	Sarstedt	72.699
2	1.5 ml Micro tubes	Sarstedt	72.690.001
3	10 ml syringes	Becton Dickinson	300613
4	15 ml Tubes	Sarstedt	62.554.502
5	2 ml Micro tubes	Sarstedt	72.695
6	50 ml Tubes	Sarstedt	62.547.254
7	96 well plates (flat bottom)	Sarstedt	84.1835
8	96 well plates (round bottom)	Sarstedt	83.1835
9	ELISA plates	Fisher Scientific	07-200-35
10	Flow cytometry tubes	Elkay	2052-003
11	Insulin syringe (with needle) 30g x 8mm	BD	324893
12	MultiScreen HTS HA, 0.45 µm, sterile	Millipore	MSHAS45
13	Pasteur pipettes	SLS	
14	Petri dishes 92x16mm	Sarstedt	82.1473
15	Pipette tips 1000µl Pipette, blue	Sarstedt	70.762
16	Pipette tips 200µl Pipette, yellow	Sarstedt	70.760.002
17	Serological pipettes 10ml	Sarstedt	86.1254
18	Serologische pipettes 25ml	Sarstedt	86.1685.00
19	Syringe filter 0.25µl	Sartorius Steidim	10331103
20	Tip biosphere 10µl white rack with filter	Sarstedt	70.1131.21
21	Tip biosphere 1000µl blue rack with filter	Sarstedt	20.762.211
22	Tip biosphere 20µl yellow rack with filter	Sarstedt	70.760.213
23	Tip biosphere 200µl yellow rack with filter	Sarstedt	70.760.211
24	Tissue culture flasks T25 Vent Cap Rad	Sarstedt	83.1810.00
25	Tissue culture flasks T75 Vent Cap Rad	Sarstedt	83.1813.00
26	Tissue culture plates 24 well plate	Sarstedt	83.1836

2.1.7 The source of plasmids used in this study

No.	Plasmid name	Source
1	Empty pcDNA3.1/ Hygro(-)	Invitrogen
2	pcDNA3.1/Hygro(-)-L.mexgp63	Rezvan PhD thesis, 2007 (NTU)
3	pcDNA3.1/Hygro(-)-Ldcen3	Asteal, PhD thesis, 2011 (NTU)
4	pCR®II-TOPO®-Ldcen1	Dr Nakhasi, FDA, USA
5	pcRT7/CT-TOPO-Ldcen3	Dr Nakhasi, FDA, USA
6	VR12-L.mexgp63	Prof. Dumonteil (Laboratorio de Parasitologia, C.I.R, Mexico)

2.2 Methods

2.2.1 Bulking of original plasmids

A sufficient amount of each DNA plasmid is required for further analysis, therefore bulking up of VR1012-L.mexgp63, pcDNA3.1/Hygro(-)-L.mexgp63 and pcRT7/CT-TOPO-Ldcen3, was performed using the standard molecular biology protocols.

2.2.1.1 The preparation of competent *Escherichia coli* cells

All *Escherichia coli* XILB strain competent cells used in this study were obtained from one single colony (Invitrogen, UK). Bulking of *E. coli* was performed in three days according to the manufacturer's guidelines and protocols.

Day one: one aliquot of *E. coli* XILB cells was thawed out on ice, and then streaked on two LB agar plates containing Tetracycline antibiotic (Sigma, UK) 5µg per ml. Plates were incubated overnight at 37°C.

On day two: one single colony was picked up and re-suspended in 3ml of fresh sterile LB broth media containing 5µg per ml Tetracycline, the suspension was then incubated overnight at 37°C with shaking at 200rpm using Stuart S150 shaking incubator.

On day three: a 500µl of *E. coli* suspension from the previous step was added into 100ml of LB broth media plus 5µg per ml Tetracycline in a conical flask. The suspension was allowed to recover in a rocking incubator at 37°C for 5 hours. *E. coli* cells were left to grow until OD600 = 0.500. Cells were then prepared for freezing as follows: the *E. coli* cells were centrifuged at 500g for 15 minutes. Supernatant was discarded and the pellet was re-suspended in 30ml of TFB I buffer (30mM of KoAc, 50 mM of MnCl₂, 100mM KCl, 10mM CaCl₂ and 15M Glycerol), pH was adjusted to pH 5.8 with acetic acid. The suspension was incubated on ice for 30 minutes, cells were then centrifuged at 500g at 4°C for 20 minutes, supernatant was removed, and the pellet was re-suspended in 4ml of buffer TFB II (100mM MOPS, 75mM CaCl₂, 10mM KCl and 15M Glycerol, pH was adjusted to pH 6.8 with KOH). Competent *E. coli* cells were aliquoted using sterile Eppendorf tubes under a sterile condition and stored at -80°C.

2.2.1.2 Bulking of original *Leishmania* DNA constructs

The bulking up of VR1012-L.mexgp63 plasmid, pcDNA3.1/Hygro(-)-L.mexgp63, pcRT7/CT-TOPO-Ldcen3, pCR®II-TOPO®-Ldcen1, pcDNA3.1/Hygro(-)-Ldcen3 and empty pcDNA3.1/Hygro(-) plasmids was also performed in two days as described in sections 2.2.1.2.1 and 2.2.2.1.2. Kanamycin or ampicillin (Sigma, UK) were used as

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a selective antibiotic, according to the plasmid maps (Figures, 2.2.1, 2.2.2, 2.2.3, and 2.2.4).

2.2.1.2.1 Day one: Transformation of plasmid DNA to the competent cells

A 200µl aliquot of *E. coli* XLIB bacteria was allowed to defrost on ice, and 1µl of DNA plasmid was gently added followed by incubation on ice for 30 minutes. The mixture was exposed to a heat-shock in a water bath at 42°C for 3 minutes, to ensure the uptake of the plasmid DNA by XLIB bacteria, followed by incubation on ice for 5 minutes. A volume of 300µl of fresh LB broth media was then added and incubated for 30 minutes at 37°C in a rocking incubator. The suspension was then transformed into fresh LB broth media supplemented with 15µg/ml kanamycin, a selective antibiotic (Figure 2.2.1), and incubated overnight at 37°C in a shaking incubator.

2.2.1.2.2 Day two: Plasmid DNA extraction

Plasmid DNA extraction was performed using a Wizard® Plus SVMinipreps Purification System (Promega, UK), protocols and guidelines of manufacturers were followed. Briefly, the suspension of XLIB bacteria broth culture was harvested using sterile 1.5 ml micro-centrifuge tubes. XLIB *E. coli* cells were centrifuged at 8000g for 5 minutes at room temperature. After the LB broth media was completely removed, the cell pellet was re-suspended in 250µl of the provided re-suspension solution. Cells were lysed by adding 250µl of Cell lysis buffer, plus 10µl of alkaline protease solution. After incubation for 3 minutes, 350µl of the Wizard® Plus SV neutralization solution was added and mixed well with the lysed *E. coli* cells. The suspension was then centrifuged at 8000g for 10 minutes at room temperature. The clear supernatant was transferred into the provided spin column, and further centrifuged for 2 minutes. This was followed by two washes using 750µl and 250µl respectively of the provided washing buffer. The ethanol was completely removed by an additional spin step for 2 minutes. Plasmid DNA was eluted in nuclease-free water and centrifuged for two minutes at room temperature. The final product was stored at -20°C.

The structure of VR1012, pcRT7/CT-TOPO, pCR®II-TOPO®, and pcDNA3.1/Hygro(-) plasmids used in this study are shown in Figures 2.2.1, 2.2.1, 2.2.3, and 2.2.3, respectively.

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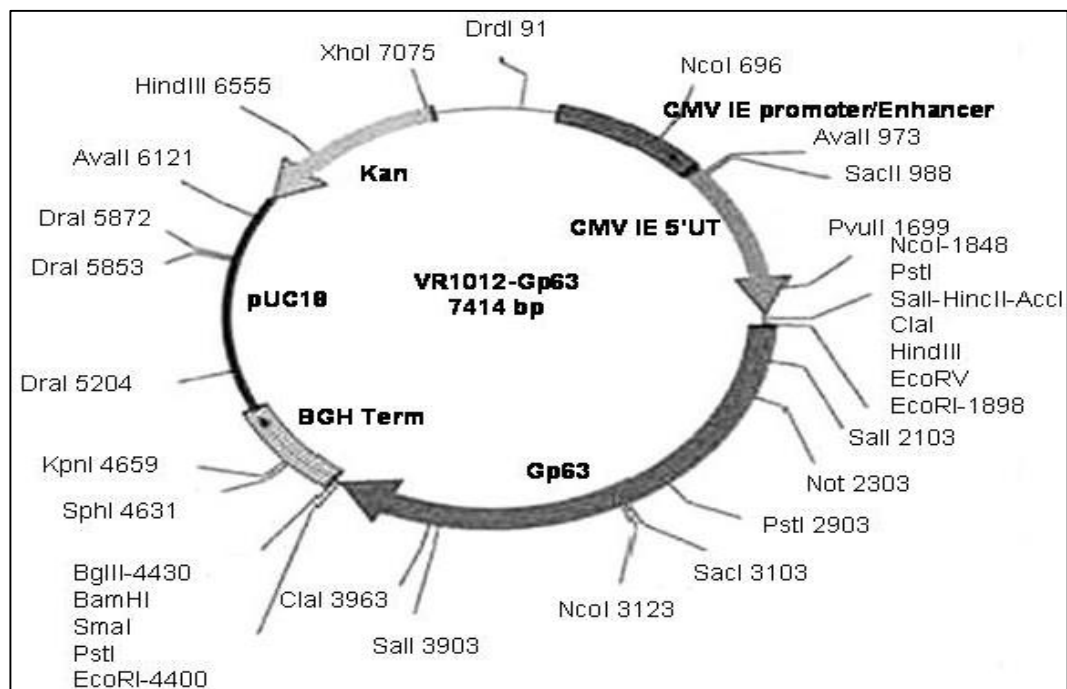


Figure 2.2-1 The structure of VR1012-L.mexgp63 plasmid map with restriction sites (Gift from Prof. Dumonteil, Mexico) *et al.*, 2003)

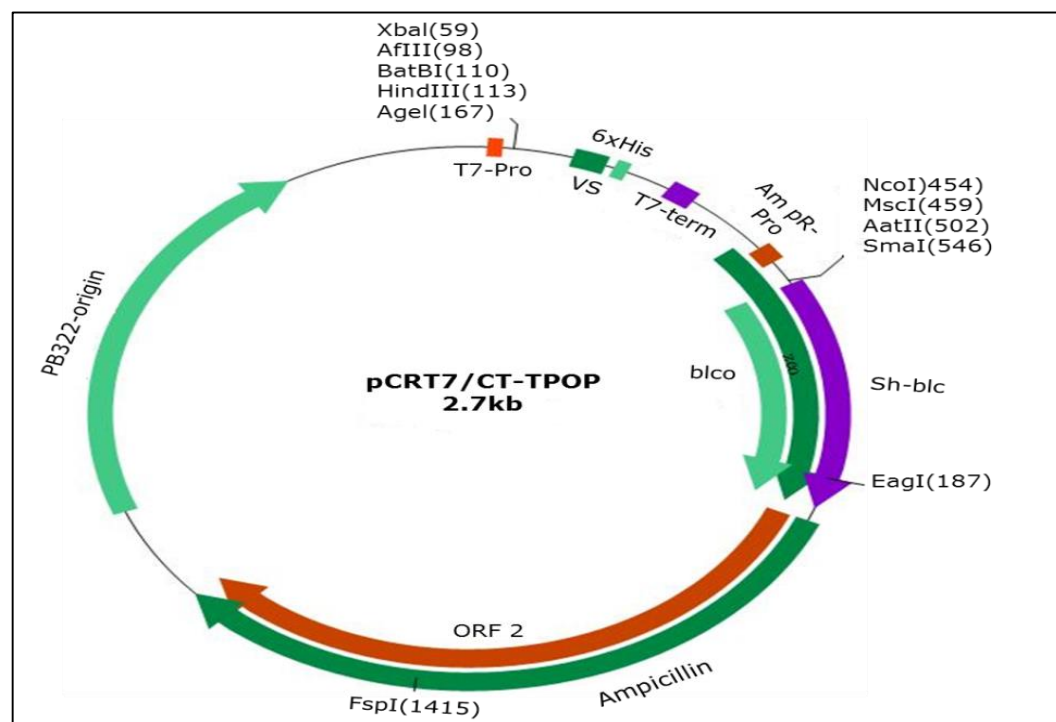


Figure 2.2-2 The structure of pCRT7/CT-TOPO plasmid with restriction sites ([http://www.biovisualtech.com/bvplasmid/pCRT7 CT-TOPO.htm](http://www.biovisualtech.com/bvplasmid/pCRT7_CT-TOPO.htm))

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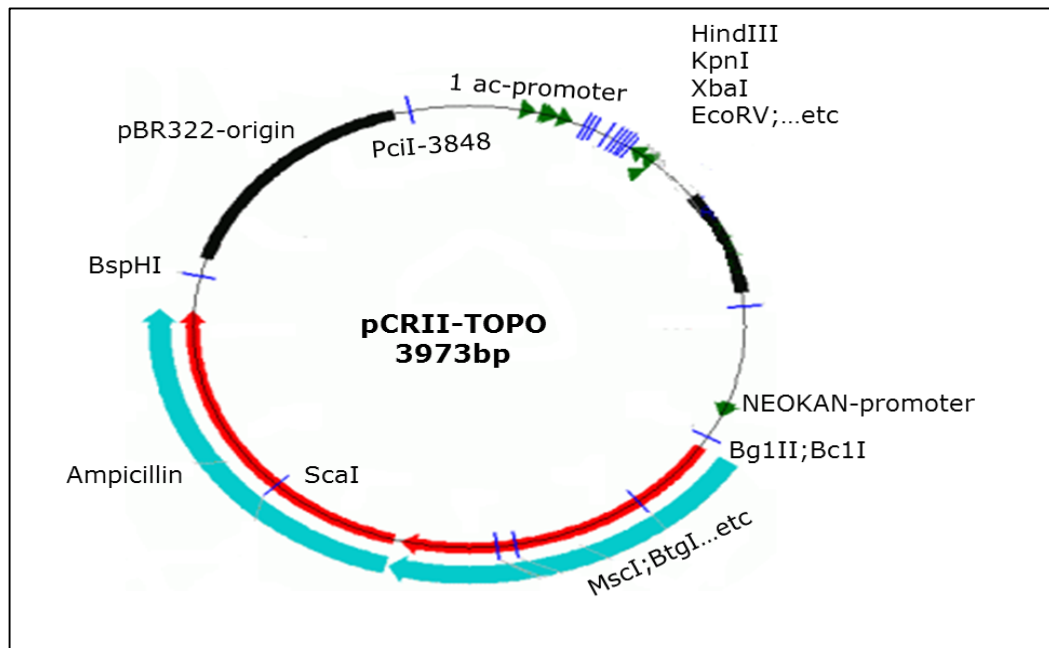


Figure 2.2-3 The structure of pCR®II-TOPO® plasmid with restriction sites
[\(http://www.addgene.org/vector-database/2289/\)](http://www.addgene.org/vector-database/2289/)

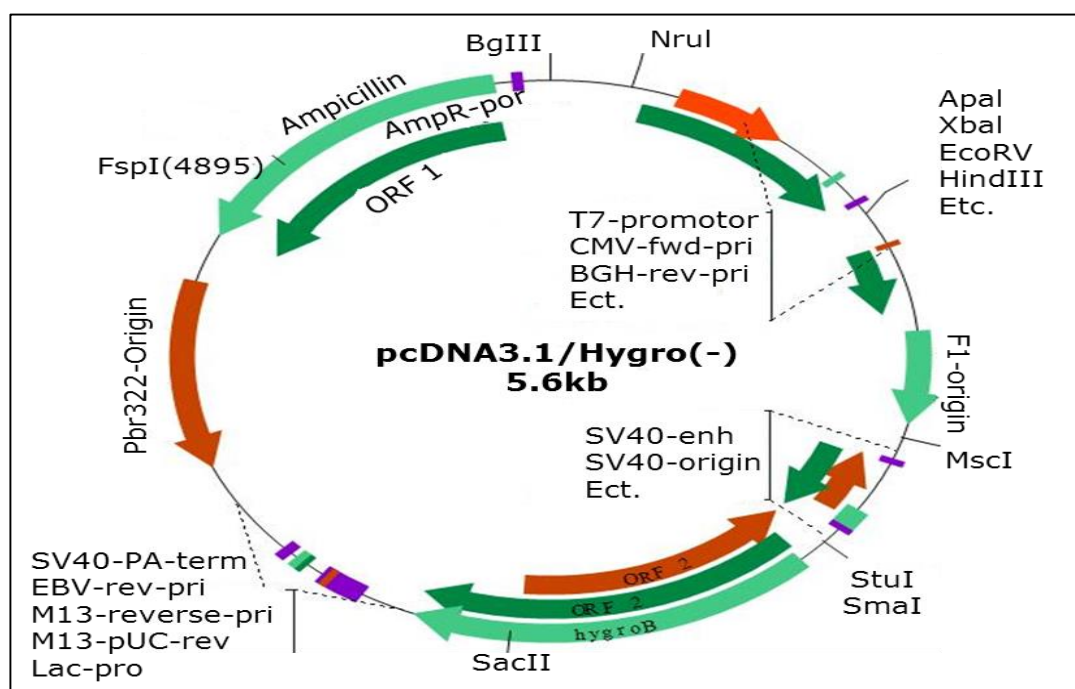


Figure 2.2-4 The structure of pcDNA3.1/Hygro(-) plasmid with restriction sites
http://www.biovisualtech.com/bvplasmid/pcDNA3.1_Hygro%28-%29.htm

2) Materials and Methods

2.2.1.3 Confirmation of the presence of *Leishmania* genes in bulked plasmids by PCR Amplification

In order to check the presence of the cloned genes (*L. mexicana* GP63, *L. donovani* centrin1 and *L. donovani* centrin3) in the bulked plasmids, these plasmids were used as the DNA template for a polymerase chain reaction (PCR). The PCR reaction was performed in a total volume of 20µl per PCR tube by adding following volumes:

Reagent	Volume (µl)
10 µM of forward primer	01.00 µl
10 µM of Reverse primer	01.00 µl
10 mM dNTPs	00.4 µl
5u/µlGoTaq® Flexi DNA Polymerase	00.2 µl
5X Green GoTaq® Reaction Buffer	04.0 µl
DNA template	02.00 µl
Nuclease-free Water	11.4µl
Total Volume	20.00 µl per tube

Table 2.2-1 List of regents and volumes used in the conventional PCR

To confirm the presence of *L. mexicana* GP63 gene in the VR1012-L.mexgp63 and pcDNA3.1/Hygro (-) plasmids, pair 1 primers (Table 2.2.2), was used. The presence of *L. donovani* centrin1 in pCR®II-TOPO® plasmid was confirmed using pair 5 primers (Table 2.2.2). The presence of *L. donovani* centrin3 in pcDNA3.1/Hygro(-)-Ldcen3 plasmid was confirmed using pair 3 primers (Table 2.2.2).

The PCR reactions were always carried out in duplicate using a Primus Advanced Thermal Cyclers PCR machine (Geneflow). The PCR reaction conditions were: initial denaturation at 95°C for 5 minutes followed by 30 cycles of 95°C for 30 seconds of denaturation, and annealing temperature was preformed according to each primer as listed in table 2.2.2. Extension step at 72°C for 1 minute, a final extension was performed at 72°C for 10 minutes, and the final hold was at 4°C for 24 hours. The PCR products were then subjected to 1.5% w/v agarose gel electrophoresis separation.

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Target gene	Primer séquence (5' → 3')	MT °C	Prod. size (bp)
1- L.mexgp63	F.P. ACATCCTCACCGACGAGAAG R.P. CTTGAAGTCGCCACAGATCA	60	194 bp
2- L.mexgp63	F.P. TCTATCTAGAATGCCCGTCGACAGC R.P. TCTAAAGCTTTCACGTCGGGAGCC	65	1940 bp
3- Ldcen 3	F.P. ACAATCAGCCGCTTATGGAC R.P. AATCATTGCCTGCAGCTCTT	58	203 bp
4- Ldcen3	F.P. TCTAGTCGACATGAACATCACTAGTC R.P. TCTAAGATCTGTAGTCGTCCTCCTCT	65	543 bp
5- Ldcen1	F.P. CTGACGGATGAACAGATTCTG R.P. AAAGCTTTCACGTCGGGAGC	60	200 bp
6- Ldcen1	F.P. ATGGCTGCGCTGACGGAT R.P. CTACTTTCCACGCATGTGC	61	450 bp

Table 2.2-2 List of primers used for PCR amplifications

The sequence of forward (F), reverse (R) primer, melting temperature (MT) and product size for each target gene. Primers were designed using an available online Primer 3PlusL software

2) Materials and Methods

2.2.1.4 Preparation of 1.5% w/v agarose gel electrophoresis

A 1.5% w/v Hi-Res Standard Agarose (Geneflow, UK) was used. Gels were stained with 5µl of Gel-Red™ Nucleic Acid Gel Stain, 10,000X in water, melted agarose was poured into a casting chamber with the appropriate comb, and left to set up at room temperature. After 30 minutes, the samples (PCR products) were loaded into gel along with 1kp DNA ladder (Promega, UK). Agarose gel electrophoresis was performed at 80 volts for 1 hour. The gel was imaged using a gel documentation system (InGenivis, UK).

2.2.2 Preparation of the new *Leishmania* DNA constructs

DNA constructs underlined in the following table were newly constructed in this study to compare immunogenicity of three *Leishmania* genes: *L. mexicana* GP63 (L.mexgp63), *L. donovani* centrin1 (Ldcen1) and *L. donovani* centrin3 (Ldcen3), in three different plasmids.

Group 1	Group 2	Group 3	Group 4
<u>pcRT7/CT-TOPO-L.mexgp63</u>	<u>pcRT7/CT-TOPO-Ldcen1</u>	pcRT7/CT-TOPO-Ldcen3	<u>Empty pcRT7/CT-TOPO</u>
VR1012-L.mexgp63	<u>VR1012-Ldcen1</u>	<u>VR1012-Ldcen3</u>	<u>Empty VR1012</u>
pcDNA3.1/Hygro(-)-L.mexgp63	<u>pcDNA3.1/Hygro(-)-Ldcen1</u>	pcDNA3.1/Hygro(-)-Ldcen3	Empty pcDNA3.1/Hygro(-)

Table 2.2-3 DNA constructs used in the current study

The underlined (red) constructs are newly prepared

2.2.2.1 The construction of pcRT7/CT-TOPO-L.mexgp63 plasmid

A new pair of primers containing the sequence of Hind III and d XbaI restriction sequences was designed (Table 2.2.2, underlined sequences). These new primers were designed to amplify the complete *L. mexicana* GP63 gene along with restriction sites, from VR1012-L.mexgp63 plasmid. PCR reaction was performed at a melting temperature of 65°C and the mixture was subjected to the same conditions mentioned in section 2.2.1.3.

Final PCR product (*L. mexicana* GP63 gene) was loaded into 1.5% w/v agarose gel (prepared as previously described) along with 1kp DNA ladder (Promega, UK). The gel was run at 80 volts for 1 hour.

2) Materials and Methods

2.2.2.1.1 Isolation of the *L. mexicana* GP63 gene fragments

The bands (*L. mexicana* GP63 gene) were cut out of the gel using a sterile scalpel and sterile Eppendorf tube under blue light transilluminator to avoid damaging the DNA. The PCR product was then purified using a Qiagen gel extraction kit according to the manufacturer's protocol. Briefly: three volumes of QG buffer were mixed with one volume of the gel. The mixture was then incubated in water bath at 50°C for 10 minutes to be dissolved completely. The mixture was mixed well with one volume of isopropanol (Sigma, UK). The samples were then transferred into the provided MinElute columns to allow the DNA to bind to their membrane by passing the sample through them. To optimise the binding of DNA, 500µl of QG buffer was further added to each MinElute column. DNA washing was performed by passing 750µl of buffer PE through each MinElute column. It was necessary that all the buffer PE was completely removed from the sample at 8000g. DNA was finally eluted in 10µl of buffer EB; samples were collected in new sterile collection tubes and stored at -20°C.

2.2.2.1.2 Restriction and digestion of *L. mexicana* GP63 gene and pcRT7/CT-TOPO plasmid

In order to increase the efficiency of the ligation step, the full length of amplified *L. mexicana* GP63 gene and the empty pcRT7/CT-TOPO were both exposed to Hind III and Xba I restriction enzymes (Promega, UK), in order to create corresponding sticky ends. The reaction was performed in a final volume of 10µl as following: 6µl of PCR product, 1µl of nuclease-free water (Qiagen, UK), 1µl of buffer E, 1µl of Hind III enzyme and 1µl of Xba I enzyme were mixed in a sterile Eppendorf tube. For plasmid digestion, 2µl of plasmid, 6µl of nuclease-free water, 1µl of buffer E, 1µl of Hind III enzyme and 1µl of Xba I enzyme were mixed in a sterile Eppendorf tube. Reaction volumes were centrifuged for 10 second, and then incubated in a water bath for 45 minutes at 37°C. Both reaction volumes were run onto 1.5% w/v agarose gel electrophoresis. Bands which represent the empty pcRT7/CT-TOPO and *L. mexicana* GP63 gene were cut out of the gel separately and DNA isolation was performed using a Qiagen gel extraction kit according to the manufacturer's protocol as described above (section 2.2.2.1.1).

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2.2.2.1.3 Dephosphorylation of plasmid DNA

Both sticky ends of digested plasmids are likely to re-ligate together, which causes reduction of the efficiency of the ligation process. Therefore, digested plasmids must be dephosphorylated to provide a highly successful ligation process. The process was achieved by using alkaline phosphatase, calf intestinal (CIAP) (Promega, UK). The reaction was performed by adding 1µl of dephosphorlation buffer and 0.5µl of alkaline phosphatase enzyme to 10µl of digested plasmid. The mixture was incubated in a water bath at 37°C for 30 minutes.

2.2.2.1.4 Ligation of *L. mexicana* GP63 gene and pcRT7/CT-TOPO plasmid

In order to insert the *L. mexicana* gp63 gene into pCRT7/CT-TOPO plasmid, they have to be mixed at a ratio of 1:3 (vector: insert) when the gene of interest is smaller than target plasmid, and at a ratio of 3:1 (vector: insert) when the gene of interest is bigger than target plasmid. The quantity of insert to be used was calculated using the following formula:

$$(\text{Ng of vector}) \times (\text{kb size of insert}) / (\text{kb size of vector}) \times \text{molar ratio of (insert/vector)} \\ = \text{ng of insert gene of interest.}$$

The amount of plasmid used was 100ng per microlitre. After calculation of the required amount from the gene of interest, this was mixed with 1µl of 10x ligation buffer and 1µl of T4 ligase enzyme (Promega, UK) and adjusted to 10µl using nuclease free water. The mixture was gently mixed and incubated over night at 4°C.

2.2.2.1.5 Transformation

The transformation of the newly prepared DNA constructs into appropriate cells was accomplished as follows:

2.2.2.1.5.1 Day 1 of transformation

pcRT7/CT-TOPO-L.mexgp63 plasmid was transformed into XLIB *E. coli* bacteria as previously described in paragraph 2.2.1.2.1. After the heat shock, *E. coli* cells were recovered in 300µl of fresh LB broth media for at least 1 hour. This was followed by growing the transfected *E. coli* suspension on two LB agar plates containing the selective antibiotic (15µg ampicillin per ml) by streaking 50µl and 150µl of both, respectively. A third plate was similarly streaked with un-ligated plasmid (linear forms) as a control. The plates were inverted and incubated at 37°C overnight.

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2.2.2.1.5.2 Day 2 of transformation

On the following day, at least five single colonies were separately selected and transferred into sterile universal tubes containing 3ml LB broth media supplemented with 15µg ampicillin per ml. The suspension was incubated overnight at 37°C in a shaker incubator, to allow the growth of *E. coli* cells to produce more copies of DNA plasmid.

2.2.2.1.5.3 Day 3 of transformation

During the last day of the transformation, pcRT7/CT-TOPO-L.mexgp63 plasmid construct was isolated from *E. coli* genomic DNA using a Wizard® Plus SVMinipreps Purification System (Promega, UK) as has been previously described in paragraph 2.2.1.2.2.

2.2.2.1.6 Confirmation of the newly cloned *L. mexicana* GP63 gene

The success of sub-cloning of *L. mexicana* GP63 gene in pcRT7/CT-TOPO plasmid was confirmed using three methods. First: the presence of the *L. mexicana* GP63 gene was determined by restriction digestion of the newly constructed pcRT7/CT-TOPO-L.mexgp63 with Hind III and Xba I restriction enzymes. Second: the presence of the *L. mexicana* GP63 gene was confirmed by PCR using pair 2 primers (Table 2.2.2). Third: the new plasmid construct was sequenced to ensure that no base miss-match occurred during the cloning procedure. Due to the length of the *L. mexicana* GP63 gene, three forward primers were used to sequence the whole gene:

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Primer Number	Primer séquence (5'→ 3')	MT °C
1	ACATCCTCAACGAGAAG	59.4°C
2	GCGATGTTGTGCAATGAGAG	57.3°C.
3	TTAAGTTGAGCAGCGTGAGC	57.3

Table 2.2-4 List of primers used to sequence *L. mexicana* GP63 gene

2.2.2.2 The construction of pcRT7/CT-TOPO-Ldcen1

The pcRT7/CT-TOPO-Ldcen3, and pCR®II-TOPO®-Ldcen1 plasmids were digested with Hind III and Xba I restriction enzymes, in order to obtain empty pcRT7/CT-TOPO and *L. donovani* centrin1. 2µl of each plasmid was digested by 1µl of each enzyme plus 1µl buffer E in a sterile 1.5 ml Eppendorf tube. The reaction volumes were adjusted to 10µl final volume with nuclease-free water. The digestion and restriction steps were carried out at 37°C in a water bath. Fragments of the digested plasmids were separated by 1.5% w/v agarose gel, and the bands representing the empty pcRT7/CT-TOPO plasmid and *L. donovani* centrin1 gene were excised from the gel. DNA was extracted from the gel using a Qiagen gel extraction kit, according to manufacturer's protocol as described previously (section 2.2.2.1.1), and the empty pcRT7/CT-TOPO plasmid was dephosphorylated as described in section 2.2.2.1.3. To perform the ligation step, the sufficient amount of DNA from the dephosphorylated vector and the insert (*L. donovani* centrin1) was calculated, using a ratio of 1:3 (vector: insert), and the ligation step was prepared as previously described in paragraph 2.2.2.1.4. A volume of 2µl from the new DNA construct (pcRT7/CT-TOPO-Ldcen1) was transformed into one aliquot XLIB *E. coli* to perform a 3-day long transformation procedure as described in sections 2.2.2.1.5.1, 2.2.2.1.5.2 and 2.2.2.1.5.3. The presence of *L. donovani* centrin1 in the new DNA construct was confirmed using three methods, as previously described.

2.2.2.3 Construction of VR1012-Ldcen1 plasmid

EcoR restriction enzyme was used to digest VR1012-L.mexgp63 and pCR®II-TOPO®-Ldcen1 plasmids in order to obtain empty VR1012 plasmid and *L. donovani* centrin1 respectively. Digestion, purification, and dephosphorylation of the empty plasmid, ligation, and transformation steps were performed as previously described in sections 2.2.2.1.2, 2.2.2.1.1, 2.2.2.1.3, 2.2.2.1.4, 2.2.2.1.5.1, 2.2.2.1.5.2 and 2.2.2.1.5.3, respectively. Since both ends of *L. donovani* centrin1 gene as well as VR1012 plasmid were digested using the same restriction enzyme, it was necessary to check that

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L. donovani centrin1 had the correct orientation. The correct orientation to the *L. donovani* centrin1 gene, which cloned into VR1012 plasmid, was checked by an enzyme restriction analysis using *Just bio* online analysis software. The success of *L. donovani* centrin1 gene into VR1012 plasmid was proven using the three methods mentioned in section 2.2.2.1.6.

2.2.2.4 The construction of VR1012-Ldcen3 plasmid

Due to the absence of the preferred restriction sites, on pcRT7/CT-TOPO-Ldcen3 plasmid required to cut off the *L. donovani* centrin3 gene, two restriction enzymes were chosen in order to design a new pair of forward and reverse primers containing the recognition sites of Sall and BgIII, respectively, pair 4 (Table 2.2.2). These new primers were used to amplify the target gene using pcRT7/CT-TOPO-Ldcen3 plasmid as a DNA template for a PCR reaction. The amplified PCR product (*L. donovani* centrin3 gene) was run *via* gel electrophoresis, and extracted using a Qiagen gel extraction kit. VR1012 plasmid was digested with Sall and BgIII restriction enzymes in order to create the complementary sticky ends. The digestion step was carried out as mentioned above. Empty VR1012 was removed from the gel and the DNA was dephosphorylated as previously described in section 2.2.2.1.3.

The digested VR1012 plasmid and *L. donovani* centrin3 gene (PCR product), were mixed together at a ratio of 1:3 (plasmid: insert). T4 ligase enzyme and 10x buffer were added to the mixture and incubated at 4°C overnight, as explained in section 2.2.2.1.4. The newly prepared VR1012-Ldcen3 DNA construct was transformed into *E. coli* cells as previously described in sections 2.2.2.1.5.1, 2.2.2.1.5.2 and 2.2.2.1.5.3. The presence of *L. donovani* centrin3 gene in VR1012 plasmid was confirmed as previously described in section 2.2.2.1.6.

2.2.2.5 The construction of pcDNA3.1/Hygro(-)-Ldcen1 plasmid

Hind III and Xba I restriction enzymes (Promega) were used to digest the empty pcDNA3.1/Hygro(-) and pCR®II-TOPO®-Ldcen1 plasmids, in order to obtain the linear form of the empty plasmid and *L. donovani* centrin1. The digested plasmids were subjected to 1.5% w/v agarose gel, and target DNA bands were excised as described in section 2.2.2.1.1. After DNA purification, the empty plasmid was dephosphorylated as previously described in section 2.2.2.1.3.

Both the *L. donovani* centrin1 gene and pcDNA3.1/Hygro(-) plasmid were ligated with a ratio of 1:3 (vector: insert), transformation and confirmation steps of newly prepared

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DNA construct (pcDNA3.1/Hygro(-)-Ldcen1) were performed as previously described for other constructs in this study.

2.2.2.6 The preparation of the empty plasmids

Restriction and digestion methods were used to prepare the empty VR1012 and pcRT7/CT-TOPO plasmids. VR1012-Lmexgp63 plasmid was digested with EcoRI restriction enzyme to remove the inserted *L. mexicana* GP63 gene, and pcRT7/CT-TOPO-Ldcen3 was digested with Hind III and Xba I restriction enzymes to remove the *L. donovani* centrin3 gene. Both of the digested plasmids were subjected to 1.5% w/v agarose gel at 80 volts for 1 hour. The bands representing each plasmid were gel extracted, and the ends of each plasmid were re-ligated using T4 ligase enzyme as described above. The obtained products were used as DNA template to confirm the absence of the removed genes by PCR, *L. mexicana* GP63 gene and *L. donovani* centrin3 gene from VR1012 and pcRT7/CT-TOPO plasmids, respectively.

2.2.3 Transfection of human cell line with DNA constructs

The prepared *Leishmania* DNA constructs (Table 2.2.2) were transfected into the MCF-7 (Michigan Cancer Foundation-7) human cell line to check the expression of these plasmids in eukaryotic cells using Lipofectamine™2000 Transfection Reagent. In the case of transfection with plasmids encoding *L. mexicana* GP63 gene, immunofluorescence staining was performed to check the expression of GP63 gene in transfected cells. Due to the lack of availability of anti-centrin antibodies, the expression of *L. donovani* centrin1 and *L. donovani* centrin3 was performed using qPCR analysis.

2.2.3.1 Antibiotic sensitivity assay

The MCF-7 cell line was obtained from Mr S. Reeder, JvGCRC, NTU, (ATCC- HTB-22™) and grown in DMEM medium (Lonza, UK) supplemented with 10% v/v FCS and 1% v/v NEAA (Lonza, UK). In order to find the optimum cell number to be used in the antibiotic sensitivity assay, firstly MCF-7 cells were seeded at serial concentrations from 1×10^2 to 1×10^5 cells per ml per well in 24 well plates. Plates were incubated at 37°C with 5% v/v CO₂ incubator for 10 days. At the end of the incubation period, 1×10^3 cells resulted in gave 80% confluent. Therefore, MCF-7 cells were cultured in 24 well plates at the concentration of 1×10^3 per ml in the presence of serial concentrations of 500, 250, 125, 75µg per ml Geneticin (G418) in duplicate. Microscopic examination revealed that the lowest concentration of the antibiotic in

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which all MCF-7 cells died in 8-10 days was 500µg per ml. A similar experiment was repeated in order to find the optimum toxic concentration of zeocin antibiotic. Cells were grown in a 24 well plate at the concentration of 1×10^3 in the presence of 100, 50, 25, 12.5 and 6.25µg per ml. Plates were kept at 37°C with 5% v/v CO₂ for 10 days, the lower concentration at which all MCF-7 cells died was 50µg per ml.

2.2.3.2 Transfection of MCF-7 tumour cells with DNA constructs

MCF-7 tumour cells were transfected with pcRT7/CT-TOPO- L.mexgp63, VR10- L.mexgp63 and pcDNA3.1-L.mexgp63 using Lipofectamine™ 2000 (Invitrogen, UK) according to the manufacturer's instruction for adherent cells with slight modifications. Briefly, MCF-7 tumour cells were cultured at 5×10^5 per well in 24-well plates, cells were allowed to adhere for 24 hours at 37°C with 5% v/v CO₂. On the following day, Lipofectamine™ 2000-DNA construct complexes were prepared using serum-free Opti-MEM medium (Lonza, UK) to increase the efficiency of complex formation. Briefly, Lipofectamine™ 2000 and the required DNA were diluted in serum-free Opti-MEM medium at 2µl/50µl and 1µg/50µl respectively, and incubated at room temperature for 5 minutes. The diluted Lipofectamine™ 2000 and DNA were mixed together and further incubated for 30 minutes at room temperature. The MCF-7 cell culture supernatant was removed and the Lipofectamine™ 2000-DNA was gently added at 100µl per well, followed by 6 hours incubation at 37°C in an incubator with a 5% v/v CO₂, after which transfected cells were recovered in 1ml of fresh media. On the following day, the culture medium was replaced with 1ml of medium containing selective antibiotic at a concentration of 500µg or 50µg for Geneticin® (G418) or Zeocin, respectively. Cells were then cultured in the presence of the selective antibiotic for at least three passages before being subjected to further analysis.

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2.2.3.3 Detection of *Leishmania* genes in transfected cells

2.2.3.3.1 Detection of *L. mexicana* GP63 in transfected cells

Immunofluorescence staining was used in this study to check for the presence of *L. mexicana* GP63 protein in cells transfected with DNA constructs encoding GP63 gene. Briefly, after three passages in the selective media, transfected cells were cultured at a concentration of 5×10^5 for 24 hours on glass coverslips inserted into 24 well plates (the coverslips were dipped into 70% v/v methanol before use). On the following day, the culture medium was removed and cells were washed twice with 1xPBS. Cells were then fixed for 15 minutes at 4°C using 500µl of 4% w/v paraformaldehyde (Sigma, UK). At the end of incubation period, the paraformaldehyde was removed and wells were washed once with 1xPBS. Coverslips were blocked with 500µl of blocking buffer (0.1% v/v Tween[®]20 and 5% w/v bovine serum albumin (BSA) in 1xPBS) for 1 hour at room temperature. Blocking buffer was then washed off using 1ml of washing buffer (0.1% v/v Tween[®]20 in 1xPBS). The monoclonal primary anti-GP63 antibody (GenWay, Switzerland) was diluted 1:500 using blocking buffer, and 300µl of diluted antibody was added to each well, plates were incubated over-night at 4°C. Control wells were left without the primary antibody (just blocking buffer was added). On the following day, the coverslips were washed, using washing buffer, three times (each wash preformed for 10 minutes shaker). Goat-anti mouse IgG2a FITC (Serotec, UK), a secondary antibody, was diluted 1:1000 in blocking buffer and added to the coverslips (300µl per well), including the controls. Coverslips were light protected and incubated at 4°C on a rocker for two hours. Finally, the coverslips were washed three times as above, and placed face down onto glass slide, and mounted with DAPI (Vector, UK), slides were visualized and imaged using fluorescence microscope (Olympus).

2.2.3.3.2 Detection of *L. donovani* centrin1 and *L. donovani* centrin3 in transfected cells by PCR

Total mRNA was extracted from transfected cells (MCF-7) using a Qiagen kit, according to the manufacturer's protocol. Extracted RNA was converted to cDNA using M-MLV (Moloney murine leukaemia virus) enzyme (Promega, UK) method following the manufacturer's protocol. Obtained cDNA was used as DNA template for qPCR, using the following primers:

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Gene name	Primer pair sequence	M°C	Pro. size (bp)
<i>L. donovani</i> centrin1	F.P. CGATGTGCTGAAGGAGATGA R.P. ACGCTCTTGAACCTCCTCGAA	60	78
<i>L. donovani</i> centrin3	F.P. GAGAGGCATTCGAGCTGTTC R.P. GCATGCTGACCTTCATCTCA	60	72

Table 2.2-5 *L. donovani* centrin primers used to detect centrin1 and centrin3 genes in transfected MCF-7 cell line

2.2.4 The immunological protocols

2.2.4.1 The preparation of soluble *Leishmania* antigens (SLA), and protein quantification

The late log phase of *L. mexicana* promastigotes M379 ($\leq 2 \times 10^7$ promastigotes per ml), growing in Schneider's *Drosophila* medium supplemented with 10% v/v HIFCS were centrifuged at 2000g at 4°C for 10 minutes. The supernatant was discarded and the pellet was washed five times with sterile 1xPBS. The pellet was re-suspended in sterile 1xPBS (500µl for 2×10^7 promastigotes) and exposed to five cycles of freezing and thawing in liquid nitrogen. The lysate was further homogenised using a Pellet Pestles (Sigma, UK). The protein concentration of the product was quantified, and kept at -80°C for further use. The total protein concentration of extracted protein was measured using a Bicinchoninic Acid Protein Assay Kit (Sigma, UK), according to the manufacturer's protocol. Briefly, a Nunc-Immuno 96 Micro Well™ Plate was loaded with serial dilutions of BSA protein (Sigma, UK) in order to create a standard curve (Appendix 5) consisting of 7 concentrations using a stock of BSA (Sigma, UK) in 1xPBS solution at a concentration of 1mg per ml. Following thorough mixing, a volume of 25µl from each concentration as well as unknown samples (SLA) were plated in triplicate. A volume of 200µl working reagent (1:50, buffer B: buffer A, respectively) was added to each well. The plate was light protected and kept for 30 minutes in a 37°C incubator. Colour development was measured using a Spectrophotometer at an absorbance of 570nm according to the manufacturer's protocol.

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2.2.4.2 Gene-gun gold bullets preparation

Plasmid constructs encoding *Leishmania* spp genes were coated onto 1.0µm gold microcarrier beads according to the manufacturer's instructions. Briefly, in order to help the DNA binding to the gold, 200µl of spermidine (Sigma, UK) was mixed with 16.6 mg of gold (Bio-Rad, UK), the solution was well mixed, and 36µg of the DNA construct was added. After 10 seconds sonication, 200µl of 1mM CaCl₂ was added drop wise to the mixture while sonicating. To precipitate the DNA, the mixture was allowed to stand for 10 minutes at room temperature. Precipitated gold-DNA complex was pelleted by centrifugation 8000g for 5 minutes, and the pellet was washed three times with 1ml of 100% v/v anhydrous ethanol (Sigma, UK). After the last wash, the gold-DNA pellet was re-suspended in 2ml of 0.025mg/ml polyvinylpyrrolidone (PVP) in a sterile 15ml conical tube. The gold PVP solution was then loaded into dried Tefzel tubes using a 5 ml syringe. The solution was left to settle for 30 minutes on the Prep Station, so the gold particles settled to the bottom of the tube. The supernatant was gently poured off using the attached syringe. The tube was rotated for 5 seconds and then left to dry by turning the N₂ gas on for 20 minutes. The plastic tubing was removed from the Prep Station and cut using a guillotine into 1 cm (bullets) that will be loaded into the gene gun. DNA Bullets were kept at 4°C until require.

2.2.4.3 Immunisation with DNA constructs

2.2.4.3.1 Animals

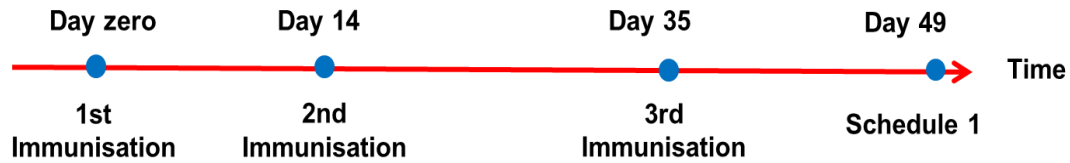
Balb/c mice were obtained from Charles River, UK and Harlan. All animals were handled in accordance with the Home Office Codes of Practice for the housing and care of animals and procedures were performed under the relevant Project Licence 40/3317, in the BSF unit at Nottingham Trent University.

2.2.4.3.2 DNA bullets immunisation

A comprehensive study was designed in order to investigate the immunogenicity of the prepared DNA constructs according to the Table 2.2.3.

Groups of 4 female Balb/c mice were immunised 3 times with 1µg DNA of each vector coated on gold particles (prepared in section 2.2.4.2), using a Gene Gun (Bio-Rad, UK) on a shaved area of the Balb/c abdomen according to following schedule:

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Two controls were considered in this study, mice immunised with empty gold bullets (just gold without any DNA), and mice immunised with empty plasmids. Fourteen days after the last immunisation, all mice were killed using carbon dioxide (CO₂) euthanasia. On the day of harvesting the following tissues were collected from each mouse.

- 1) Blood samples were collected by cardiac puncture and serum prepared for the analysis of the presence of antibody responses (total IgG, IgG2a and IgGb) using Enzyme-linked immunosorbent assay (ELISA).
- 2) Spleens were collected and splenocytes prepared for the analysis IFN- γ secretion, proliferation assay and CD4⁺, CD8⁺ profiling using flow cytometry analysis. Supernatants from the functional assays were collected for the determination of IL-2, IL-4 and IFN- γ levels by ELISA.
- 3) Lymph nodes were collected and lymph node cells prepared for the analysis of proliferation and CD4⁺, CD8⁺ profiling using flow cytometry analysis. Supernatants from the functional assays were collected for the determination of IL-2, IL-4 and IFN- γ levels by ELISA.
- 4) Bones were collected to generate bone marrow-derived macrophage (BM-DM).

2.2.4.4 Preparation of splenocytes suspension

Spleens from immunised and control mice were harvested under sterile conditions. The spleen bags containing the splenocytes were flushed out using serum-free RPMI 1640 medium using a 25g needle and 5 ml syringe. The splenocyte suspensions were transferred into 15ml tube and centrifuged at 800g for 5 minutes at 4°C. The supernatant was aspirated, and the pellet was re-suspended in 3ml of T cell medium (RPMI 1640 medium supplemented with 10% v/v FCS, 20mM HEPES, 50 μ M 2 Mercaptoethanol, 50 μ M per ml penicillin-streptomycin and 0.25 μ g per ml Fungizone). Cells were counted using trypan blue solution, and cultured at different cell number concentrations according to the need of each assay.

2.2.4.5 Preparation of lymphocytes suspension

Ten lymph nodes from each mouse were collected and placed into 1.5 Eppendorf containing 1 ml of PBS. Under sterile conditions, the lymph nodes were washed three

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times with serum-free medium, and homogenised using disposal pellet pestles (Sigma, UK). The suspension of lymphocytes was washed twice before counting using trypan blue solution. Lymphocytes were cultured in RPMI 1640 medium supplemented with 10% v/v FCS, 20mM HEPES, 50µM 2 Mercaptoethanol, 50µM per ml Penicillin-Streptomycin and 0.25µg per ml Fungizone.

2.2.4.6 IFN γ Elispot assay

The Enzyme-Linked ImmunoSpot (Elispot) assay was used in the current study to detect the secreted IFN- γ by splenocytes of immunised mice in response to *in vitro* stimulation with *L. mexicana* Soluble Antigen (SLA). The Elispot assay was preformed according to the manufacturer's protocol with minor modifications. Briefly, a day before harvesting, MultiScreen® plates were pre-wetted with 100µl of 35% v/v ethanol for 1 minute. Each plate was then washed three times with sterile phosphate buffered saline (PBS) after tapping it against a clean tissue paper to remove any residual liquid. The required mouse IFN- γ capture antibody was diluted 1:60 in PBS and loaded onto the Elispot plate (100 µl per well), and incubated overnight at 4°C. On the day of harvesting, the capture antibody solution was removed, and the plates were washed three times with sterile PBS and blocked with C.T.L-Test™ Medium (Cellular Technology Ltd, UK) for 2 hours at 37°C in a 5% v/v CO₂ incubator. Splenocytes from immunised mice and controls were seeded at a concentration of 1×10^6 in 100µl of T cell medium. Cells were stimulated with another 100µl per well of fresh media contain 10µg SLA per ml. Plates were then incubated at 37°C in an incubator with a 5% v/v CO₂ atmosphere. After 48 hours, plates were re-stimulated with 30 and 50µg per ml SLA and incubated for a further 24 hours. The test was performed in triplicate for each concentration. A positive control was also used for each plate using Staphylococcal enterotoxin B (SEB) (Sigma, UK) at a concentration of 20µg per ml. At the end of the incubation period, culture medium was removed, and the Elispot plates were washed four times using wash buffer (0.05% v/v Tween®20 in PBS), and wells were loaded with 100µl of mouse IFN- γ detection antibody diluted in filtered reagent diluent (1% w/v BSA in PBS, pH 7.2-7.4). Plates were covered and incubated overnight at 4°C. On the following day, the plates were developed using Elispot Blue Color Module (R&D system, UK). Briefly, the detection antibodies were completely removed from the plates by three washes with washing buffer, and a further three washes with 1xPBS, followed by three washes with distilled water. Plates were blotted against a clean tissue paper,

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and loaded with 100µl of Streptavidin-AP diluted 1:60 in reagent diluent, then, protected from the light were incubated for 2 hours at room temperature. The plates were then washed three times with wash buffer and distilled water, then loaded with provided BCIP/NBT solution (100µl per well) and incubated for 30 minutes at room temperature. Plates were then washed with deionized water and allowed to dry in the dark at room temperature. Developed spots were counted automatically using an Elispot reader (Cellular technology Limited, UK).

2.2.4.7 CD4 and CD8 profiling by flow cytometric analysis

In order to monitor the effect of the *Leishmania* DNA constructs on the percentage ratios of CD4⁺ and CD8⁺ in the spleens and lymph nodes of the immunised mice and controls, 10⁶ cells of splenocytes and lymphocytes per FACS[®] tube were stained for their expression of CD4⁺ and CD8⁺ and analysed using flow cytometry. Briefly, cells were placed in FACS[®] tubes at a concentration of 1x10⁶ cells per tube. Cells were then washed twice with two ml of FACS buffer (1xPBS containing 0.1% w/v BSA plus 0.1% NaN₃) at 400g and 4°C for 5 minutes. Both rat-anti mouse CD4: FITC and rat-anti mouse CD8: RPE (Serotec, UK) were diluted 1:10 in FACS buffer, and 10µl of diluted antibody were simultaneously added to the test FACS tubes. Relevant isotopes, rat IgG2a negative control: FITC and rat IgG2a negative control: RPE (Serotec, UK) were treated similarly and added to isotype tubes. Cells were light protected and incubated for 20 minutes at room temperature. Cells were then washed twice with 2 ml of FACS buffer. After the last wash, cells were re-suspended in 250µl of Isoton, and analysed using a Beckman Coulter Gallios, 10 colour flow cytometer (Beckman Coulter, UK). The results were presented as percentage of positive CD4 and/or CD8 compared to the whole population of splenocytes or lymphocytes.

2.2.4.8 Proliferation assay

A proliferation assay was used in this study to analyse responses to immunization with the gene gun. The increase in DNA synthesis as result of splenocytes proliferation was measured by adding [³H]-Thymidine, a radioisotope-DNA labelling agent. Briefly splenocytes and lymphocytes were counted and plated at a concentration of 5x10⁵ in 100µl of T cells medium per well in a round bottomed 96-well plate. Cells were stimulated with 10µg per ml SLA in 100µl of culture media (just culture medium without SLA was added to the control wells). Plates were then incubated at 37°C in a 5% v/v CO₂ incubator. 72 hours later, the splenocytes and the lymphocytes were re-

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stimulated with 30 and 50µg SLA per ml and plus [³H] Thymidine 1µ Ci per well. The plates were further incubated for 72 hours at 37°C in 5% v/v CO₂ incubator. The test was performed in triplicate for each concentration. At the end of the incubation period, plates were harvested onto a 96 well UniFilter GF/C plate, using Filtermate Harvester (Packard), and left to dry for a few hours at room temperature. Newly synthesised DNA was measured using TopCount Nxt™ (Packard, UK).

2.2.4.9 ELISA assays (IFN-γ, IL-4 and IL-2)

ELISA (Enzyme-linked immunosorbent assay) approaches were used to estimate the concentration of IFN-γ, IL-4 and IL-2 cytokines. Splenocytes and lymphocytes (from spleens and lymph nodes, respectively) were cultured at a concentration of 10⁶ per well per ml of T cells medium using 24 well plates. Two doses of SLA were used to stimulate the cells, 30 and 50µg SLA per ml. Plates were then incubated at 37°C in 5% v/v CO₂ incubator. Supernatant (900µl) was collected from control and test wells at 2 time points (day 3 and day 5) of the culture. Samples were kept at -20°C until analysis for cytokine levels. The levels of IFN-γ, IL-4 and IL-2 in the culture supernatant of splenocytes and lymphocytes were determined using commercially available Deu-Set ELISA Kits (R&D System, UK). The assays were carried out following the manufacturer's instructions.

2.2.4.10 Detection of anti-*Leishmania* IgG, IgG2a and IgG1 isotype antibodies

On the day of harvesting, blood samples from immunised and control mice were collected as described in section 2.2.4.3.2. The blood samples were stored at 4°C for eight hours, and the serum was harvested from each sample by centrifugation at 8000g for 15 minutes. The serum was collected and stored at -20°C. Total IgG, IgG1 and IgG2a was estimated by ELISA. Briefly, a flat-bottomed 96-well plate (Fisher Scientific, UK) was coated with 100µl per well of coating buffer (50mM sodium carbonate buffer, pH 9.6) containing a 5µg per ml *L. mexicana* SLA. The plates were sealed and incubated overnight at 4°C. After 3 washes with washing buffer (1x PBS and 0.05% v/v Tween 20), the plates were blocked with blocking buffer (1% w/v casein in 1xPBS) for 1 hour at 37°C in an incubator. Plates were washed 4 times with washing buffer, and 100µl per well of 1:100 dilution of the serum samples in 1xPBS were added. They were then sealed and incubated at room temperature for 2 hours. Plates were washed 4 times with shaking for 1 minute per wash using washing buffer. Goat anti-

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mouse IgG2a: HRP (Serotec, UK), Rabbit anti-mouse IgG1: HRP (Serotec, UK), or Goat anti-mouse IgG (Abcam, UK) were diluted at 1:8000, 1:100000, and 1:100000 (antibody: blocking buffer) respectively were added at a volume of 100µl per well. Plates were light protected and incubated at room temperature for 1 hour. At the end of the incubation period, the plates were washed as described above, and 100µl of substrate solution (1: 1 reagent A: reagent B) was added to each well. Plates were incubated for 20 minutes at room temperature. The reaction was stopped by adding 50µl of 2.5M H₂SO₄, and the absorbance (OD) was measured at 570 nm by spectrophotometer.

2.2.4.11 The generation of bone marrow-derived macrophages (BM-DMs)

Hind limbs of naïve and immunised Balb/c mice were collected on the day of harvesting and used to generate the adherent bone marrow-derived macrophages (BM-DMs), according to Inaba, *et. al.*, (1992) protocol. Briefly, tissues and muscles were completely removed using scalpel and tweezers under sterile conditions. Bone-marrow cells were flushed out after cutting the ends of the bones. Cells were then centrifuged at 400g at 4°C for 5 minutes, and the pellet was re-suspended in 2ml of fresh BM-DC medium. Cell counting was performed using 0.4% w/v trypan blue stain in phosphate buffered saline (Sigma, UK), and plated in 24 well plates at a concentration of 10⁶ cells per well per ml of fresh media supplemented with 1µg per ml of Granulocyte-macrophage colony-stimulating factor (GM-CSF), (Sigma, UK). Plates were then incubated at 37°C in 5% v/v CO₂ under 95% v/v humidity. On days 2 and 4, BM-DCs were washed by gently changing 75% of old medium (containing non-adherent DCs) with fresh DC medium containing GM-CSF. On day 6, adherent BM-DM were gently detached using Pasteur pipettes and seeded in 24 well plates at a concentration of 2x10⁵ cells per well. 4-6 hours later cells were pulsed with 1µg Lipopolysaccharides (LPS), (Sigma, UK), and incubated at 37°C in 5% v/v CO₂ under 95% v/v humidity for 24 hours.

Before processing, the obtained macrophages were phenotypically characterised for the expression of specific mouse macrophage marker (F4/80) and dendritic cells CD11c marker by flow cytometric analysis. Using the Serotec rat anti-mouse F4/80: FITC and its relevant isotype IgG2b, and Hamster anti-mouse CD11c: FITC and its relevant isotype IgG antibodies.

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2.2.4.12 Survival assay

A new assay to measure the susceptibility of BM-DMs from controls and immunised mice to *Leishmania* infection was established for the first time in this study. Matured BM-DMs from naïve and immunised mice were infected with late log phase of virulent *L. mexicana* promastigotes at a ratio of 1:10 (cell: parasite). Infected cells were incubated under 95% v/v humidity at 37°C in a 5% v/v CO₂. Six hours after the infection, free promastigotes were removed by changing the medium, and infected cells were further incubated under the same conditions for 48 hours. Afterwards, infected macrophages were first detached using Pasteur pipettes, and then homogenized by passing them through a 0.25G needle at least five times in order to obtain a suspension of single *L. mexicana* amastigotes. The homogenised BM-DMs were completely removed by centrifugation of the homogenized cell suspension at 1000g and 4°C for 10 minutes. The supernatant, which contained the amastigote stage, was transferred into new sterile 15ml tubes, which were further centrifuged at 3000g and 4°C (to pellet the amastigote stage) for 15 minutes. The supernatant was then discarded and the pellet was washed three times using sterile PBS re-suspended in 5 ml of Schneider's *Drosophila* medium supplemented with 20% v/v HIFCS in a T25 tissue culture flask. 5x10⁵ amastigotes from each sample of infected macrophages (generated from controls and immunised mice), were then kept at 25°C and monitored for promastigote stage recovery for up to two weeks. A 50µl of culture medium (supernatant) from each well for each mouse was collected at 30 minutes, 24 and 48 hours to be used for IFN-γ estimation using the ELISA method and for the measure of nitrite levels using standard Griess Reagent System according to the manufacturer's protocol.

2.2.4.12.1 IFN- γ and nitrite concentration

The level of IFN- γ in culture media was measured using commercial Deu-Set IFN- γ ELISA Kits (R&D System, UK) according to the manufacturer's protocol. Standard curve was produced for each experiment (Appendix 6). Measurement of nitrite in the culture medium was performed using Griess Reagent System (Promega, UK) with all the samples were immediately subjected to nitrite concentration analysis after harvesting. Briefly, 50 μ l of experimental sample was plated into a 96 well plate (Sarstedt, UK) then the test was carried out in triplicate. 50 μ l of the provided Sulfanilamide solution was added to each well, and the plate was then light protected and incubated at room temperature for 10 minutes. After the first incubation time, 50 μ l of NED solution was added to all samples, and the plate was also light protected and further incubated for an extra 10 minutes at room temperature for colour development. Absorbance was measured using an Asys UVM 340 Microplate Reader (Biochrom) at 495nm. The standard reference curve (Appendix 8), for quantification of nitrite concentration was performed for each experiment as follows. The provided 0.1M nitrite standard was diluted 1:1000 in RPMI medium in order to prepare 1ml of 100 μ M nitrite solution, then serially diluted to give 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.0 μ M nitrite solution.

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2.2.5 *L. mexicana* infectivity and susceptible human cell lines

2.2.5.1 Cell lines and parasite

2.2.5.1.1 MonoMac-6 cell line

The human monocytic cell line MonoMac-6 was obtained from DSMZ-German (DSMZ no. ACC124). MonoMac-6 cells were cultured in a complete RPMI medium, supplemented with 10% v/v FCS, 2mM L-Glutamine, 1mM Na pyruvate, 1% v/v non-essential amino acids, 9µg/ml bovine insulin and Penicillin-Streptomycin 200µg/ml. Cells were maintained at a concentration of $1-5 \times 10^5$ cells/ml using 25 or 75cm³ tissue culture flasks, under 95% v/v humidity at 37°C in a 5% v/v CO₂ incubator. Cultured cells were checked and maintained regularly.

2.2.5.1.2 U937 monocytes cell line

The human U937 monocyte cell line was a kind gift from Prof. SJ Forsythe, Pathogen Research Group, NTU. U937 monocyte cells were cultured according to *ICLC*, in RPMI 1640 supplemented with 10% v/v FCS, 2mM L-Glutamine and 200µg/ml of Penicillin-Streptomycin. Cells were grown under 95% v/v humidity at 37°C in a 5% v/v CO₂ incubator. Cells were sub-cultured regularly every 2-3 days to keep them at a concentration of $3-5 \times 10^5$ cells per ml.

2.2.5.1.3 W6/32HK Hybridoma cell line

Mouse hybridoma W6/32 cell line was obtained from European Culture Collection (Catalogue No. 84112003). Cells were maintained in RPMI supplemented with 10% v/v FCS and 2mM glutamine in a T25 tissue culture flask, under 95% v/v humidity at 37°C in a 5% v/v CO₂ incubator. In order to collect the supernatant containing anti-human HLA (MHC I) antibodies from the culture of W6/32, cells were seeded in 5 ml of fresh media per T25 flask and cultured until they became 80% confluent. The old culture medium was replaced by 5 ml of fresh medium, and cells were incubated for at least 2 weeks before supernatant-containing antibodies (mouse monoclonal mouse anti-HLA antibodies) were collected. Supernatants were centrifuged at 400g and 4°C for 3 minutes, supernatant was passed through a 0.25µm filter for sterilisation, and stored at -80°C.

2.2.5.1.4 Maintaining the *Leishmania mexicana* parasite

The *Leishmania mexicana* (MNYC/BZ/62/M379) strain was obtained from Dr V. Yardley, The London School of Hygiene and Tropical Medicine (LSHTM). The

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virulency of *L. mexicana* promastigotes was maintained by continuously passaging them in susceptible Balb/c mice. Groups of three Balb/c mice were injected intradermally with 2×10^6 stationary phase *L. mexicana* promastigotes in a total volume of 50 μ l per mouse in sterile 1xPBS. Mice were injected in a shaved area at the back about 1cm from the tail base. The maintenance and care of mice were according to the guidelines of the Home Office. Mice were killed when they developed the required lesion. After two passages of amastigotes in *Drosophila* Schneider media (Lonza, UK), supplemented with 10% v/v HIFCS at 25°C, obtained promastigotes were further cultured and frozen in liquid nitrogen for future work.

2.2.5.2 The genetic identification of *L. mexicana* parasite

The obtained parasites were genetically confirmed as *L. mexicana* species, by Restriction Fragment Length Polymorphism analysis (RFLP), using the method of Schonian, *et. al.*, (2003) method, with minor changes. First, the genomic DNA was extracted from late log *L. mexicana* promastigote culture using an InstaGene™ Matrix kit (Bio-Rad, UK) according to the manufacturer's instructions. Briefly, 200 μ l of late log parasite culture was pelleted at 2000g for 5 minutes, and the pellet was washed three times with sterile 1xPBS, and then re-suspended in 30 μ l of autoclaved nuclease free water. 20 μ l of the parasite lysate was mixed with 200 μ l of InstaGene™ matrix, and incubated at 56°C for 30 minutes. The suspension was then vortexed and incubated at 100°C using a heat block for 8 minutes. The product was stored at -20°C to be used as DNA template. Ribosomal internal transcribed spacer (IST1) region was amplified by PCR from extracted genomic DNA. The PCR reaction was set up in a 20 μ l final volume as follows: 1 μ l of 10 μ M OL1853 primer (CTGGATCATTTTCCGATG), 1 μ L of 10 μ M OL1854 primer (TGATACCACTTATCGCACTT), 1 μ l of genomic DNA, 4 μ l of 5xGreen GoTaq® Reaction Buffer, 0.2 μ l of GoTaq® Flexi DNA Polymerase, 0.4 μ l 10 mM dNTPs and 11.4 μ l nuclease-free water. The reaction was carried out in duplicate using a Primus Advanced Thermal Cyclers PCR machine (Geneflow, UK). PCR conditions were as following: initial denaturation at 95°C for 5 minutes followed by 40 cycles of 95°C for 40 seconds denaturation, 53°C annealing temperature for 40 seconds, extension step at 72°C for 50 seconds, final extension at 72°C for ten minutes, the final hold was at 4°C for 24 hours. The obtained PCR product was digested with Hae III enzyme (Promega, UK) for 45 minutes at 37°C, and the digested product was loaded into 2% w/v agarose gel electrophoresis for 2 hours.

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2.2.5.3 *L. mexicana* promastigote growth characteristics

2.2.5.3.1 The effect of *in vitro* passaging on growth curves

The *L. mexicana* parasite was derived from freshly harvested lesions from infected Balb/c mice by culturing the homogenised lesion in Drosophila Schneider media (Lonza, UK), supplemented with 10% v/v HIFCS (Fisher, UK) at 25°C. *L. mexicana* promastigotes from passage 1 (P1) were further cultured at a concentration of 1x10⁵ cells per ml up to passage 20 (P20), the parasite was grown under an anaerobic conditions in unvented flasks, this was done by properly ceiling the flask with parafilms (the efficacy ceiling was compared with culturing the parasite under strict bacterial anaerobic conditions). To count the parasite, 10µl material from the culture was fixed with 2% w/v paraformaldehyde and counted using a Neubauer hemocytometer. The growth curves of the parasite at passages 1, 7 and 20 were performed using 3 cultures for each passage number in each experiment, which was initiated at a concentration of 1x10⁵ promastigotes per ml. Experiments were repeated 3 times using different frozen aliquots. To monitor the morphological changes that were accruing over the culture period, 100µl from each flask at the late log stage, at each passage number was subjected to cell size and flagellum length measurement using a calibrated micrometer slide. Parasites with straight flagellum were measured to determine the average length of their bodies and flagella (Appendix 16).

2.2.5.3.2 The effect of *in vitro* passaging on virulence-associated genes

In order to examine and follow up the changes that occurred in the gene expression profiles of some of the genes related to parasite infectivity during their *in vitro* passaging, quantitative polymerase chain reaction qPCR analysis was used. The total mRNA from metacyclic promastigotes at P1, P7 and P20 was extracted. Briefly, 1x10⁸ *L. mexicana* promastigotes from each passage number was pelleted at 2000g for 10 minutes, followed by five washes with sterile PBS. The washed pellet was subjected to RNA extraction using Qiagen kit according to manufacturer's protocol as described in section 2.2.5.6.1. RNA quality and quantity were checked by 1.5% w/v agarose gel (Appendix 9.B), and Nano Drop®8000 Spectrophotometer (Appendix 11). Since mRNA is unstable and easily degraded, it was necessary to reverse transcribe (RT) the single strand mRNA to complementary DNA (cDNA).

2.2.5.3.3 cDNA Synthesis

Since mRNA is unstable and easily degraded, it was necessary to reverse transcribe (RT) the single strand mRNA to complementary DNA (cDNA). The synthesis of single-stranded cDNA was performed using M-MLV reverse transcriptase (Promega, UK), following the manufacturer's protocol. Each reaction was carried out in duplicate, under sterile conditions. 1µg of mRNA was diluted in 13µl of nuclease-free water and incubated at 70°C in a block heater with 1µl (0.5µg per µl) Oligo (dT)₁₅ primer for 5 minutes to denature the RNA secondary structure. The mixture was incubated on ice for 5 minutes to allow the primers to anneal to the mRNA. A mixture of 5µl of 5x reaction buffer, 1µl of 10mM dNTPs, 0.7µl of 25 Units rRNasin[®] ribonuclease inhibitor and 1µl of 200 units M-MLV RT was added to each tube of the annealed primer/template, and the total volume was adjusted to 25µl using nuclease-free water. The tubes were mixed and incubated at 39°C for 80 minutes. The synthesised cDNA was heated up to 90°C for 5 minutes to de-activate the enzymes. Quality and quantity of synthesised cDNA was determined using gel electrophoresis and a Nanodrop 8000 Spectrophotometer, respectively. The product was stored at -20°C.

qPCR analysis was performed using specific primers for amplification of each gene. Primers were designed using online Primer3Plus software according to their sequence given by the National Centre for Biotechnology Information (NCBI). The names, accession numbers of the chosen genes, Primer pair sequence, product size and melting temperatures are listed in (Table 2.2.6).

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Gene name	Accession No.	Primer Sequence	Prod. size	MT °C
histone H4	XM-003879078	F.P.TCTCGAGCGACATCTACGAA R.P.CGCACAATATCCTCCACGTA	110 bp	57.3
L.dcen	AF406767	F.P.ACGATGTGCTGAAGGAGATG R.P.CAGCATCACGCTCTTGAAC	186 bp	60
L.mex Cht1	AY572789	F.P.TCAGGACTCGTTCCACTACG R.P.ACCTCGGCACTGAAGAAGAT	72 bp	58
L.mex CPB2	AJ319727	F.P.ACGTGTTGATCGGAAGCAG R.P.CAGCGTGGTTCACCTCTTTA	147 bp	60
L.mex CPB2.8	Z49962	F.P.CGTGCCTGCTCAGTGAATAC R.P.GATCACCTGCTCCACCATC	116 bp	60
L.mex CPC	Z48599	F.P.GTGACAACGTCGAGATGGAG R.P.TACACCTGCATGGCTACCTC	124 bp	60
L.mex gp63-C1	X64394	F.P.ACCGTCTGAGAGTCGGAAC R.P.GTAGTCCAGGAATGGCGAGT	113 bp	60
L.mex LPG2	AJ278970	F.P.CATTTGGTATCCTGGTGCTG R.P.GAGGAAGCCACTGTTTAGCC	120 bp	58
L.mex- α tubulin	DQ450541	F.P.CTATCTGCATCCACATTGGC R.P.ACTTGTCAGAGGGCATGGA	111 bp	60
L.mex- β tubulin	M23441	F.P.GCGCTTTCTTCACTGGTACA R.P.TGGTACTGCTGGTACTCGGA	65 bp	60
LACK	AF363976	F.P.GGTCGTACATCAAGGTGGTG R.P.GACCGTAGTCGCTGTCCAC	96 bp	60
Sherp	XM-003875691	F.P.GACAATGCGCACAACAAGAT R.P.GTCCTTGATGCTTTCAATCG	100 bp	57.3

Table 2.2-6 List of *L. mexicana* virulence-associated genes
Primer pair sequence, accession number, product size and melting temperature (MT) of the primers used in qPCR

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2.2.5.3.4 The effect of *in vitro* passaging on *L. mexicana* GP63 protein expression

The effect on GP63 protein in *L. mexicana* promastigotes due to *in vitro* culturing was examined by Immunofluorescence staining. The promastigotes from late log phase culture of passages 1, 7 and 20 were subjected to immunofluorescence staining using anti *L. major* GP63 antibody (GenWay, Switzerland). Briefly, *L. mexicana* promastigotes (1×10^6) from the late log stage of each passage number, and late log stage of *L. major* promastigotes was used as positive control. *L. major* and *L. mexicana* promastigotes from each passage number were pelleted, washed twice with 1xPBS, and fixed in 1% w/v paraformaldehyde for 30 minutes at room temperature. The samples were then treated with 0.1M glycine (Sigma, UK) for 10 minutes at room temperature. The parasites were then washed with 500µl of PBS, and re-suspended in 100µl of 1xPBS. A volume of 50µl of the parasite suspension was placed on polystyrene tissue culture treated glass slides, and incubated overnight at 4°C (to allow parasite attachment). On the following day the slides were blocked with 200µl per well of TB buffer (0.1% w/v BSA in 1xPBS with 0.1% v/v Triton X100) for 10 minutes. The primary anti-*L. major* GP63 antibody was diluted in TB buffer, 200µl added to each well (no antibody was added to control wells), and incubated over-night at 4°C. The primary antibody was then washed off three times with 1xPBS, 200µl per well of diluted goat anti-mouse IgG2a FITC (Serotec, UK) in TB buffer (secondary antibody) was added, and the slides were light protected and incubated for 1 hour at room temperature. The slides were then washed three times with 1xPBS mounted with DAPI (Vector, UK), examined and imaged using a fluorescence microscope (Olympus).

2.2.5.3.5 The effect of *in vitro* passaging on *in vivo* infectivity of *L. mexicana*

Groups of three Balb/c mice were inoculated in a shaved area at the back about 1 cm from the tail base with 2×10^6 of late log phase *L. mexicana* promastigotes at passage 1 (P1), passage 7 (P7), or passage 20 (P20) in a total volume of 50µl per mouse in sterile 1xPBS. Mice were kept under standard conditions and monitored regularly for lesion development. In this study *L. mexicana* promastigotes P1 and P20 will be called, for simplicity, virulent and avirulent parasites, respectively.

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2.2.5.4 *L. mexicana* promastigotes-host cell interactions

2.2.5.4.1 Host cells

In addition to the use of MonoMac-6 and U937 cell lines as monocytes (sections 2.2.5.1.1, and 2.2.5.1.2), U937 monocytes were induced to differentiate into human macrophage-like cells using Phorbol 12-myristate 13-acetate (PMA, Sigma, UK) (Kim, *et. al.*, 1998). In this study, the ability of LPS (Sigma, UK) to induce U937 macrophages was also examined. Briefly, U937 monocytes were plated in 24-well tissue culture plates at a concentration of 2×10^5 cells per well per ml, using the culture medium described in section 2.2.5.1.2, and validation experiments were performed to determine the optimum concentration to be used. Briefly, four concentrations of 1, 2 and 5 μg per ml of PMA or LPS were used to mature U937 cells for 48 hours. Macrophage maturation was monitored by the expression of human macrophage CD14, CD68 and F4/80 markers using qPCR and flow cytometric analysis.

2.2.5.4.2 Characterisation of the U937 macrophage cell line

Three methods were used to confirm the maturation of U937 monocytes into U937 macrophages: Firstly, U937 macrophages were subjected to mRNA extraction using RNA kit (Qiagen, UK). The obtained mRNA was converted to cDNA, which was used as template for qPCR analysis to check the gene expression of the (F4/80, CD14, and CD68) macrophage markers. Although the F4/80 primers were initially designed for mouse *Emr1*, they amplified the human *Emr1* gene due to the high similarity between the two genes, (Table 2.2.7).

Secondly, the maturation of U937 monocytes was confirmed by staining induced macrophages with antibodies to CD14, CD68. (Biolegend, UK) and F4/80 (Serotec, UK) and analysing these by flow cytometry.

Thirdly, the phagocytic ability of matured U937 cells (macrophages) was assessed by a bead up-take assay compared to U937 monocytes. Here, U937 cells were cultured in the presence of 1, 2 and 5 $\mu\text{g}/\text{ml}$ of PMA per ml for 48 hours. U937 macrophages were mixed with 1 μl latex beads (Sigma, UK) and cells were further incubated for 24 hours. On the following day, cells were washed at least three times using sterile PBS and fixed with 2% w/v paraformaldehyde for 5 minutes. Coverslips (macrophages) or smears (monocytes) were mounted with DAPI (Vector, UK), up-taken beads by U937 macrophages and U937 monocytes were counted using a Leica TCS SP2 confocal microscopy.

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Gene Name	Accession Number	Primer pair Sequence	Product size	MT °C
CD14	NM-000591	F.P. CACAGGTTCTGCTCAGCTA R.P. AGCTGGAAAGTGCAAGTCCT	144 bp	60
CD68	NM-01040059	F.P.GCGGTGGAGTACAATGTGTC R.P.GTGGACAGCTGGTGAAAGAA	144 pb	60
F4/80	NM-010130	F.P.TTGGCCAAGATTCTCTTCCT R.P.TCACTGCCTCCACTAGCATC	127 bp	58

Table 2.2-7 Primers of human macrophage markers

Gene names, accession numbers, primer pair sequence, product size and melting temperature (MT°C) of the primers used for characterisation of U937 macrophages

2.2.5.4.3 *In vitro* infection of U937 monocytes and U937 macrophages cell lines with *L. mexicana*

U937 monocytes and U937 macrophages cells were infected with the late log phase of *L. mexicana* promastigotes at a ratio of 1:30 (target: parasite). Briefly, target cells were plated in 24 well plates at a concentration of 2×10^5 per well using fresh medium. The required virulent or avirulent *L. mexicana* promastigote numbers were washed 3 times in sterile 1xPBS and used to infect target cells, which were incubated at 37°C, 95% v/v humidity in a 5% v/v CO₂ incubator.

2.2.5.4.4 *L. mexicana* promastigote infectivity

After 2 hours of incubation, host cells were separated from the host cells by slow centrifugation at 400g for 5 minutes. The numbers of un-attached (free) virulent and avirulent *L. mexicana* promastigotes were quantified using a Neubauer Hemocytometer.

2.2.5.4.5 Inhibition of *L. mexicana* promastigote growth by conditioned medium

Human target cells (U937 monocytes or U937 macrophages) were plated and infected as described in section 2.2.5.4.3. The medium from controls and cells infected with virulent or avirulent parasites for 2 or 24 hours was collected and centrifuged at 2500g for 10 minutes to remove infected cells and the parasites. The supernatant was sterilised by passing it through a 0.25µm filter and used to investigate the inhibition effect on parasite growth. Virulent or avirulent *L. mexicana* promastigotes were cultured in 5 ml of conditioned media at a concentration of 1×10^5 per ml for three days. At the end of

2) Materials and Methods

incubation period, the number of promastigotes per ml was evaluated using a Neubauer hemocytometer.

2.2.5.4.6 Live imaging and Giemsa staining

Live imaging of infected target cells was performed using a Nikon eclipse TS100 microscope connected to Nikon digital net camera DN100 at different time points at 40x magnification. Free (un-attached) virulent or avirulent promastigotes, and released amastigotes were counted in four fields using at least six wells for every experiment. In other experiments, U937 monocytes and U937 macrophages were infected with late log phase virulent or avirulent *L. mexicana* promastigotes at a ratio of 1:10 (cell: parasite). After 2, 24 and 48 hours of infection at 37°C, infected cells were washed and fixed using 2% v/v paraformaldehyde for 5 minutes at room temperature. 20µl of the suspension of infected cells and controls was used to prepare microscope slides. After drying at room temperature, slides were stained in 5% w/v Giemsa for 3 minutes followed by an extensive wash with distilled water. The infected cells were visualized at 40x or 100x magnification.

2.2.5.5 Flow cytometric analysis of MHC class I expression in human cell lines

Flow cytometry analysis was used to determine the MHC class I expression in human cell lines after *in vitro* infection with *L. mexicana* promastigotes. The procedure was carried out over two days. On day 1, the target cells were infected with *L. mexicana* promastigotes; on day 2, infected cells were stained with specific antibodies.

2.2.5.5.1 Infection of target cells with *L. mexicana* parasites

In order to investigate the effect of different growth stages of *L. mexicana* on the MHC class I expression in infected human cells, MonoMac-6 and U937 monocytes cell lines were infected with virulent or avirulent parasites at different stages in their growth curve (Table 2.2.8), at a ratio of 1:10 (target: parasite). Infected cells were incubated in 95% v/v humidity at 37°C in a 5% v/v CO₂ incubator for 24 hours.

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Growth phase name	No. of <i>L. mexicana</i> promastigotes
Early log phase	$\geq 5 \times 10^6$ cells per ml
Mid log phase	$\geq 1 \times 10^7$ cells per ml
Late log phase	$\leq 2.5 \times 10^7$ cells per ml

Table 2.2-8 Phases of the *L. mexicana* growth curve

(Adapted from Protocols for handling and working with *Leishmania* species, Mottram, 2008)

2.2.5.5.2 Staining with antibodies for flow cytometry analysis

To determine the MHC class I expression by flow cytometry analysis, staining of infected cells was performed in a two-step protocol, firstly, staining with primary antibodies, secondly: staining with secondary antibodies. Briefly, the supernatant of the mouse W6/32 HK hybridoma cell line was used as a primary antibody. Infected cells were harvested and centrifuged for 3 minutes at 400g and 4°C and treated with 2% w/v paraformaldehyde for 5 minutes at room temperature, to kill the parasites. Infected cells were washed three times with 2ml of FACS buffer (1xPBS containing 0.1% w/v BSA plus 0.1% NaN₃) at 400g and 4°C for 3 minutes, and the number of infected cells was adjusted to a concentration of 2×10^5 per FACS[®] tube. Cells were then treated with FcR Blocking Reagent (Miltenyi, UK), for 15 minutes at room temperature. This was followed by washing with 2ml of FACS buffer at 400g and 4°C for 3 minutes. Extracellular staining of MHC class I was performed by re-suspending the cells (test tubes) in 50µl supernatant of W6/32HK hybridoma cell line as primary antibody prepared in section 2.2.5.1.3, and incubated for 15 minutes at room temperature. Cells were then washed in FACS buffer three times and stained with a 5µl per tube of diluted (1:100) rat-anti mouse:FITC (Serotec, UK) as a secondary for 15 minutes at room temperature. Stained cells were washed twice with 2 ml of FACS buffer, and the pellet was re-suspended into 300µl of Isoton. The samples were later analysed using Gallios, 10 colour flow cytometer (Beckman Coulter, UK)

2.2.5.5.3 Study of *L. mexicana* growth stages infectivity

To study the growth stage infectivity, *L. mexicana* at early and late log phases were scanned for their virulence-associated gene expression using qPCR. Briefly, a number of 1×10^8 *L. mexicana* promastigotes from early and late log phases (the number of promastigotes was either $\geq 5 \times 10^6$ or $\leq 1 \times 10^7$ per ml, respectively) were centrifuged and subjected to mRNA extraction followed by cDNA synthesis. Expression of the

2) Materials and Methods

following genes was determined: *L. mexicana* GP63, *L. mexicana* LPG2, *L. mexicana* CPC, *L. mexicana* CPB2, *L. mexicana* CPB2.8 and *L. mexicana* CHT1. The accession numbers, primer sequences, product sizes and melting temperature for each primer are listed in table 2.2.4. In other experiments, *L. mexicana* promastigotes from early and late log phases were used to infect the target cells as described above, and monitored for amastigote performance.

2.2.5.5.4 The effect of infection of target cells with high dose of *L. mexicana* promastigotes on MHC class I expression

Late log stages of virulent and avirulent *L. mexicana* promastigotes were used to infect U937 monocytes at a ratio of 1:20, 1:30, 1:50 and 1:70 for 24 hours as described above. Infected cells were stained for the expression of the MHC class I and analysed by flow cytometer.

2.2.5.5.5 Treatment of infected cells with Fungizone

In order to prove whether the induced down-regulation of MHC class I expression by *L. mexicana* promastigotes infection was reversible or not, infected cells with virulent and avirulent parasite were treated with Fungizone (Lonza, UK), an anti-*Leishmania* reagent that caused the death of the parasites by increasing their plasma membrane permeability. A validation experiment was carried to determine the optimum concentration of Fungizone used in this study. Briefly, 10^6 *L. mexicana* promastigotes per ml of medium containing serial dilutions of 2, 4, 6, 8 and 16 µg per ml Fungizone, were incubated at 25°C. Cell viability was examined under a light microscope every 30 minutes. The dose of 4 µg per ml of Fungizone was found to be the optimum concentration to kill *L. mexicana* promastigotes in 60 minutes. Host cells (U937 monocyte and U937 macrophages) were cultured at a concentration of 2×10^5 per well in 24 well plates and infected with the virulent and avirulent late log phase of *L. mexicana* at a ratio of 1:30 (cell: parasite). Cells were divided into 4 groups: the first group was used as a control, the second group was fixed two hours after infection with 2% w/v paraformaldehyde, the third group was treated with 4 µg per ml Fungizone 2 hours after the infection and further incubated for 24 hours, and a fourth group was infected for 24 hours. Infected cells were incubated in 95% v/v humidity at 37°C in a 5% v/v CO₂ incubator. The following day, controls and infected cells were subjected to MHC class I expression as described above. In other experiments, target cells were

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either treated with 4 µg per ml Fungizone 24 hours after virulent and virulent parasites infected, or infected for 48 hours. The expression of target markers was analysed by flow cytometry. All flow cytometry data were analysed using Beckman Coulter Kaluza software. .

2.2.5.6 The effect of *L. mexicana* promastigotes infection on the gene expression profile of susceptible human cell lines

In order to determine the effect of *L. mexicana* infection or their antigens on the gene expression profile of human monocyte and macrophage cells, target cells were either infected with a ratio of 1:30 (cell: promastigotes) late log phase of virulent and avirulent *L. mexicana* promastigotes, or stimulated with 10µg per ml virulent or avirulent *L. mexicana* antigens (SLA). Total mRNA was extracted from controls as well as from infected or stimulated U937 monocytes, U937 macrophages, and MonoMac-6 cell.

2.2.5.6.1 Preparation of infected target cells for RNA extraction

Before RNA extraction, infected cells were harvested and free parasites were removed by centrifugation. Adherent U937 macrophages were harvested using trypsin solution. 500µl of trypsin solution was added to each well, plates were kept for 5 minutes at 37°C in 5% v/v CO₂. The detached U937 macrophages, U937 monocytes, and MonoMac6 (infected or stimulated and controls), were collected, washed with PBS, and subjected to RNA extraction.

2.2.5.6.2 RNA extraction using Qiagen kit and cDNA synthesis

Total mRNA was extracted using a Qiagen kit, according to the manufacturer's protocol. A number of 5x10⁶ each target cells were infected with virulent or avirulent *L. mexicana* promastigotes or stimulated target cells with or their SLA, were washed three times with sterile PBS and the cell pellet was re-suspended in 350µl of buffer RLT and vortexed, cells were then homogenised using a Pellet Pestle (Sigma, UK) for 30 seconds. Homogenised cells were mixed with 350µl of 75% v/v ethanol (Sigma, UK), and transferred into the provided spin column. Columns were then centrifuged at 8000g for 30 second at room temperature. To avoid DNA contamination, samples were subjected to a DNA digest step using RNAase-Free DNase Set (Qiagen, UK). RNA was allowed to bind to a silica membrane using 350µl of buffer RW1 and incubated for 3 minutes. The buffer RWI was discarded by centrifuging the columns for 2 minutes at 8000g. 80µl of buffer RDD was gently loaded onto the silica membrane and columns

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were incubated for 15 minutes at room temperature. Buffer RDD was removed with 350µl of buffer RW, and this was spun at 8000g for 1 minute. The columns were removed and placed into new 2ml collection tubes and cleaned by two wash steps using 500µl of buffer RPE at 8000g. The bound RNA was eluted in Rnase-free water (30 µl per column) for 5 minutes. To increase the RNA yield, the last step was repeated. RNA was collected in a sterile 1.5 Eppendorf and kept at -80°C for further investigations. RNA quality and quantity were checked by 1.5% w/v agarose gel (Appendix 9.A), and Nano Drop®8000 Spectrophotometer (Appendix 10), respectively. The synthesis of single-stranded cDNA was performed using the M-MLV reverse transcriptase method (Promega, UK), following the manufacturer's protocol as described in section 2.2.5.3.3.

2.2.5.6.3 Quantitative Real-Time PCR (qPCR) analysis

The quantitative polymerase chain reaction (qPCR) technique was used to analyse and compare the modulation of target cell gene expression profile as result of infection with virulent and avirulent *L. mexicana* or their antigens. A 0.5µl of cDNA from control, infected and stimulated cells, was used to prepare the qPCR reaction in a final volume of 12.5µl, as stated in the following table:

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Reagent	volume
10 μ M forward primer	0.5 μ l
10 μ M reverse primer	0.5 μ l
2x iQ TM SYBR Green Supermix (Bio-Rad)	6.75 μ l
cDNA template	0.5 μ l
Nuclease-free water	4.25 μ l
Total volume	12.5 μ l

Table 2.2-9 The volume of each reagent used to prepare the master mix of qPCR

All qPCR reactions were prepared in duplicates using strip tubes and caps from Corbett® Rotor-Gene®. The gene expression profile of the toll-like receptor (TLR-1, TLR-2, TLR-4 and TLR-9), Chemokines (CCL-1, CCL2, CCL-3, CCL-4, CCL-5 and CCL-22), and cytokines (TNF- α , IL-1, IL-6, IL-10, IL-12 β , and TGF- β), in control, infected, or stimulated target cells were investigated. Gene accession numbers, primer pair sequences and product sizes are shown in Table (2.2.10). Three housekeeping genes used in this study are: beta-2-microglobulin (B2M), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta actin (ACTB).

All reactions were performed using a Rotor TM 6000 Real Time PCR machine (Corbett Research). Reaction conditions were as follows: initial denaturation step at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing (depending on the primers MT, table 2.2.10) for 30 seconds and an extension step at 72°C for 20 seconds. A ramp temperature between the melting temperature and 72°C was used to obtain the melting curves.

The comparative threshold method (C_t) was used in order to quantify the results obtained by qPCR. In this method, C_t values of genes of interest were compared with the C_t values of endogenous housekeeping genes. For each gene of interest the relative expression was calculated as $2^{-\Delta\Delta C_t}$.

2) Materials and Methods

Target gene	Accession number	Primer pair sequence	MT°C	Prod. size
ACTB	NM-001101	F.P.GGCATGGGTCAGAAGGATT R.P.AGAAGGTGTGGTGCCAGATT	59	136 bp
B2M	NM-004048	F.P-CATCCAGCAGAGAATGGAAA R.P-CCAGTCCTTGCTGAAAGACA	60	144 bp
CCL-1	NM-002981	F.P.ATGCAGGTACCCTTCTCCAG R.P.AACACAGGATTGCCCTCAG	60	74 bp
CCL-2	NM-002982	F.P.GCAGAAGTGGGTTCAGGATT R.P.TGGGTTGTGGAGTGAGTGTT	60	63 bp
CCL-22	NM-002990	F.P.GGCGTGGTGAAACACTTCTA R.P.GGCACAGATCTCCTTATCCC	60	100 bp
CCL3	NM-002983	F.P.CCCGGTGTCTATCTCCTAAC R.P.GCTCCAGGTCGCTGACATA	60	91 bp
CCL-4	NM-002984	F.P.AGTCTGTGCTGATCCCAGTG R.P.TGTCTCATGGAGAAGCATCC	60	128 bp
CCL-5	NM-000579	F.P.CGGTGTCTGAAATGAGAAGAA R.P.TAGGGAGCCCAGAAGAGAAA	58.3	86 bp
GAPDH	J02642	F.P.ATCATCCCTGCCTCTACTGG R.P.GTCAGGTCCACCACTGACAC	60	122 bp
IL-10	M57627	F.P.GATGCCTTCAGCAGAGTGAA R.P.ACCCTTAAAGTCCTCCAGCA	60	93 bp
IL-12B	NM-002187	F.P.GCCGTTCAACAAGCTCAAGTA R.P.GCCGAGAATTCTTTAATGGC	58.3	112 bp
IL-1 β	NM-000576	F.P.AAGCTGAGGAAGATGCTGGT R.P.CGTTATCCCATGTGTCTGAAG	60	115 bp
IL-6	NM-000600	F.P.TCAGCCCTGAGAAAGGAGAC R.P.CCATCTTTGGAAGGTTTCAGG	60	94 bp
TGF- β	NM-00066	F P ACAATTCCTGGCGATACCTC R P ACAACTCCGGTGACATCAAA	60	85 bp
TLR-1	NM-003263	F.P.ACTTATTTGGAATGGCCCAA R. P .TTTGCTTGCTCTGTCAGCTT	58.3	95 bp
TLR-2	NM-003264	F.P.CTTCACTCAGGAGCAGCAAG R.P.GTCCTGTGACATTCCGACAC	60	135 bp
TLR-4	U88880	F.P.TGGACCTGAGCTTTAATCCC RP.GTCTGGATTTCACACCTGGAT	58.3	100 bp
TLR-9	AF245704	F.P.CCAGCTACATCCCGATACCT R.P.GTTCTCACTCAGGTCCAGCA	60	136 bp
TNF- α	M10988	F P.CCCATGTTGTAGCAAACCCT R P. TGAGGTACAGGCCCTCTGAT	60	99 bp

Table 2.2-10 List of genes and primers sequence used to determine the host cells immune response

Genes, primer pair sequences, accession numbers, product sizes and melting temperatures (MT) of the primers used in qPCR

2.3 Statistical analysis

The experiments in this study were undertaken at least three times unless it is stated. Results are expressed as means \pm SEM. Data were analysed using Prism 6.0 (GraphPad Software). Statistical significance was performed using two-way ANOVA, a nonparametric Mann-Whitney U test, or a Student's t-test.

Chapter 3/ Immunisation with *Leishmania*

DNA Constructs

3. Immunisation with *Leishmania* DNA constructs

3.1 Introduction

Most of the available chemotherapy to treat Leishmaniasis, are associated with considerable resistance and side effect problems, therefore an effective vaccine is still the best available option. Recently, DNA vaccination of many diseases, including Leishmaniasis has been widely investigated in the last few decades.

The control of *Leishmania* parasite infection is a complicated and cumbersome issue, and is greatly affected by the genetic background of the host immune system, the *Leishmania* spp, and even the sand fly, which delivers the parasite into the host. For example, the resistance of C57BL/6 mice to *L. major* infection is correlated to the Th1 immune response, which characterized by a high production of IFN- γ . In contrast, the susceptibility of Balb/c mice to the same parasite is correlated to the Th2 immune response, which is characterized by a high IL-4 and IL-10 production (Louis, *et. al.*, 1998). The role of CD4⁺ and CD8⁺ T cells in protection against *Leishmania* spp has been determined through their production of IFN- γ , which in turn activates the production of NO in infected macrophages (Bogdan and Röllinghoff, 1998; Woelbing, *et. al.*, 2006). The Th1/Th2 paradigm in resistance and susceptibility to *Leishmania* infection, respectively, has been shown to be species-dependent (McMahon and Alexander, 2004; Alexander and Bryson, 2005). This increases the challenge of producing a homologous *Leishmania* vaccine. The results of many trials to develop a protective vaccine against Cutaneous and Visceral Leishmaniasis have shown to be mostly dependent on the vaccine type, and the delivery method. For example, Petters *et. al.*, (2009), have reported that vaccination with autoclaved *L. major* antigen and CpG oligodeoxynucleotides was protective against needle challenge but, failed to protect against infection with sand fly challenge.

Recently, it has been also demonstrated that the protection induced by using DNA vaccine might be vector-dependant, since pcRT7/CT-TOPO-Ldcen-3 induced better protection than pcDNA3.1-Ldcen-3 (Asteal, 2011). Centrin is a calcium binding cytoskeletal protein which is essential for duplication and segregation of centrosomes (Selvapandiyan, *et. al.*, 2007). The physiological role of the centrin protein in *Leishmania* spp has been reported by Selvapandiyan, *et. al.*, (2001) who illustrated that

3) *Leishmania* DNA constructs immunogenicity

there was high expression of centrin protein during the exponential phase of the parasite growth, and that there is less expressed at the late log stage, which indicates the importance of these proteins in parasite growth. This was further confirmed in the work of Selvapandiyan and colleagues (2004) who knocked out the centrin gene of the *L. donovani* promastigote, and demonstrated that parasite growth was affected. The immunogenicity of some *Leishmania* genes such as centrins and GP63, which are represented in almost all studied *Leishmania* spp in DNA vaccines was investigated in this study.

The capacity of DNA vaccines to protect against VL as well as CL has been determined, as were their effects in different experimental models. The variable results obtained were influenced by the type of delivery and the antigen used. The *L. donovani* LACK gene has been cloned into the pcDNA3.1 plasmid, and shown to stimulate IFN- γ secretion from lymph node cells of immunised Balb/c mice, but not to protect against systematic infection of *L. donovani* (Melby, *et. al.*, 2001). In contrast, the same plasmid (pcDNA3.1) encoding *L. major* LACK (pcDNA3.1-LmLACK) using the same method of immunisation, protects immunised mice against *L. major* (Gurunathan, *et. al.*, 1997). Melby, *et. al.*, (2001), have reported that the failure of pcDNA3.1-LdLACK to protect against *L. donovani* challenge, in comparison to pcDNA3.1-LmLACK, protection against *L. major* was not due to the difference in the amino acid sequence of the LACK in *L. donovani* and *L. major*.

In another study the immunogenicity of the VR1012 plasmid encoding *L. mexicana* GP63, CPB, LACK, and *L. amazonensis* GP46 was analysed in Balb/c mice. Although all constructs induced a humoral immune response, only VR1012-GP46, VR1012-GP63 and VR1012-CPB produced some protection as characterized by lesion size and parasite burden. Combining three genes together has been shown to produce better protection in immunised mice (Dumonteil, *et. al.*, 2003). As shown above variation in the outcome of DNA vaccine immunisation, and these could be strongly related to the delivery plasmid, or to the cloned antigen.

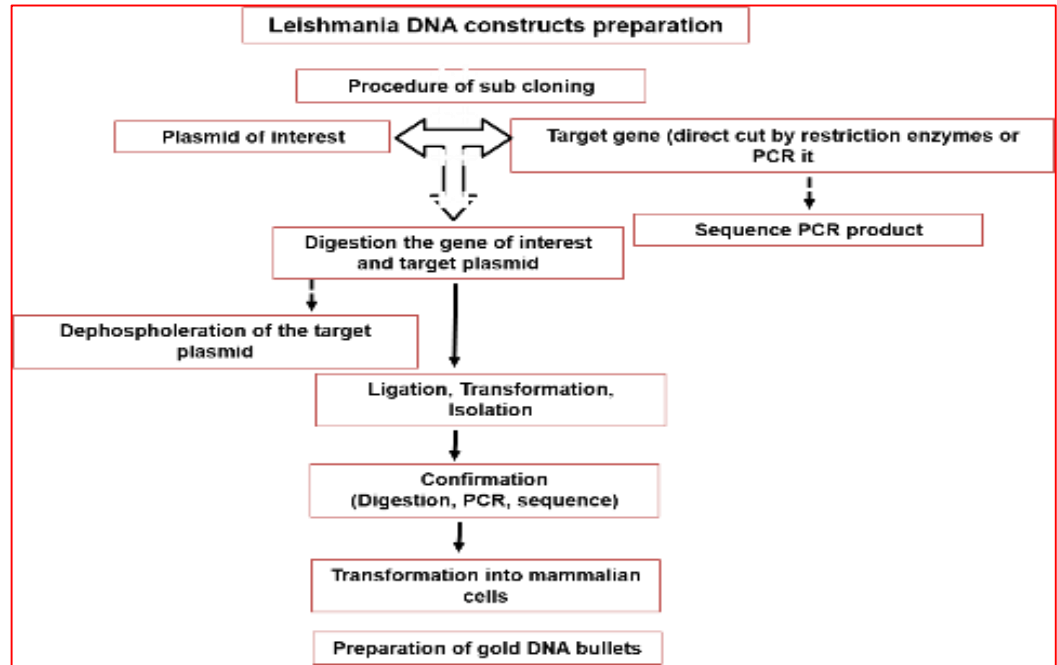
Therefore, the first aim of this study was to prepare and compare the immunogenicity induced by different plasmids encoding different *Leishmania* genes using gene gun immunisation of susceptible Balb/c mice.

3) *Leishmania* DNA constructs immunogenicity

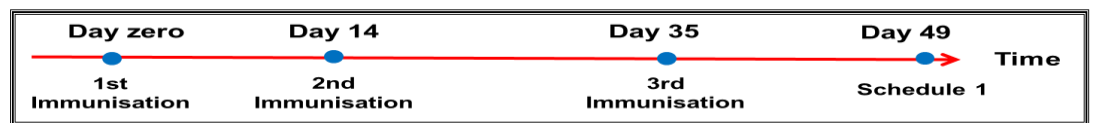
The work flow

The experiments of this chapter were carried out according to the following schedule:

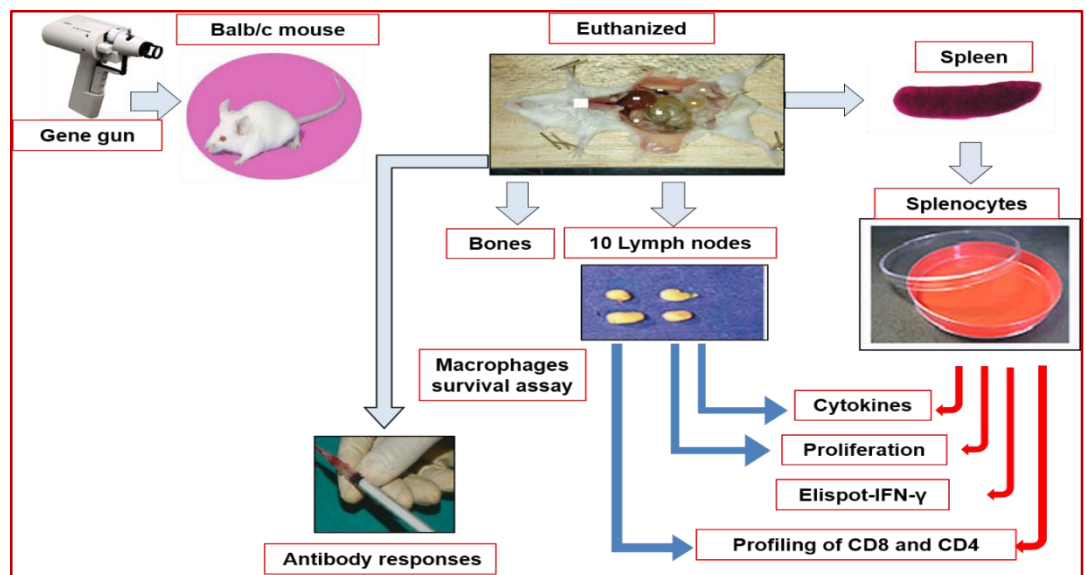
1) Preparation of *Leishmania* DNA constructs



2) Immunisation schedule



3) Immune analysis following vaccination



3.2 Cloning of *Leishmania* genes in DNA constructs

Three different plasmids containing 3 *Leishmania* genes (*L. mexicana* GP63, *L. donovani* centrin1 and *L. donovani* centrin3) were successfully cloned into three different plasmids (pcRT7/CT-TOPO, VR1012 and pcDNA3.1/Hygro(-)) in order to generate nine *Leishmania* DNA constructs. Three of those were already available in our laboratory, and the presence of target gene in each of them was confirmed as described below.

3.2.1 Confirmation of *Leishmania* genes in plasmid constructs

The VR1012-L.mexgp63 plasmid (kindly obtained from Prof. Dumonteil, C.I.R, Mexico), was bulked up by the transformation of *E. coli* bacteria, and the presence of the *L. mexicana* GP63 gene was confirmed by PCR using pair 1 primers (Figure 3.2.1.A). pcDNA3.1/Hygro(-)-L.mexgp63, which was prepared by a previous PhD student (Rezvan, PhD thesis, 2007, NTU), was also bulked and the presence of the *L. mexicana* GP63 gene was confirmed by PCR using pair 1 primers (Figure 3.2.1.B). Similarly, the presence of Ldcen1 in pCR®II-TOPO®-Ldcen1 plasmid was confirmed by PCR (Figure 3.2.1.C). The *L. donovani* centrin3 gene was successfully amplified by PCR from pcDNA3.1/Hygro-Ldcen3 DNA construct which was also prepared by a previous PhD student (Asteal, PhD thesis, 2011, NTU), using pair 3 primers (Figure 3.2.1.D).

3) *Leishmania* DNA constructs immunogenicity

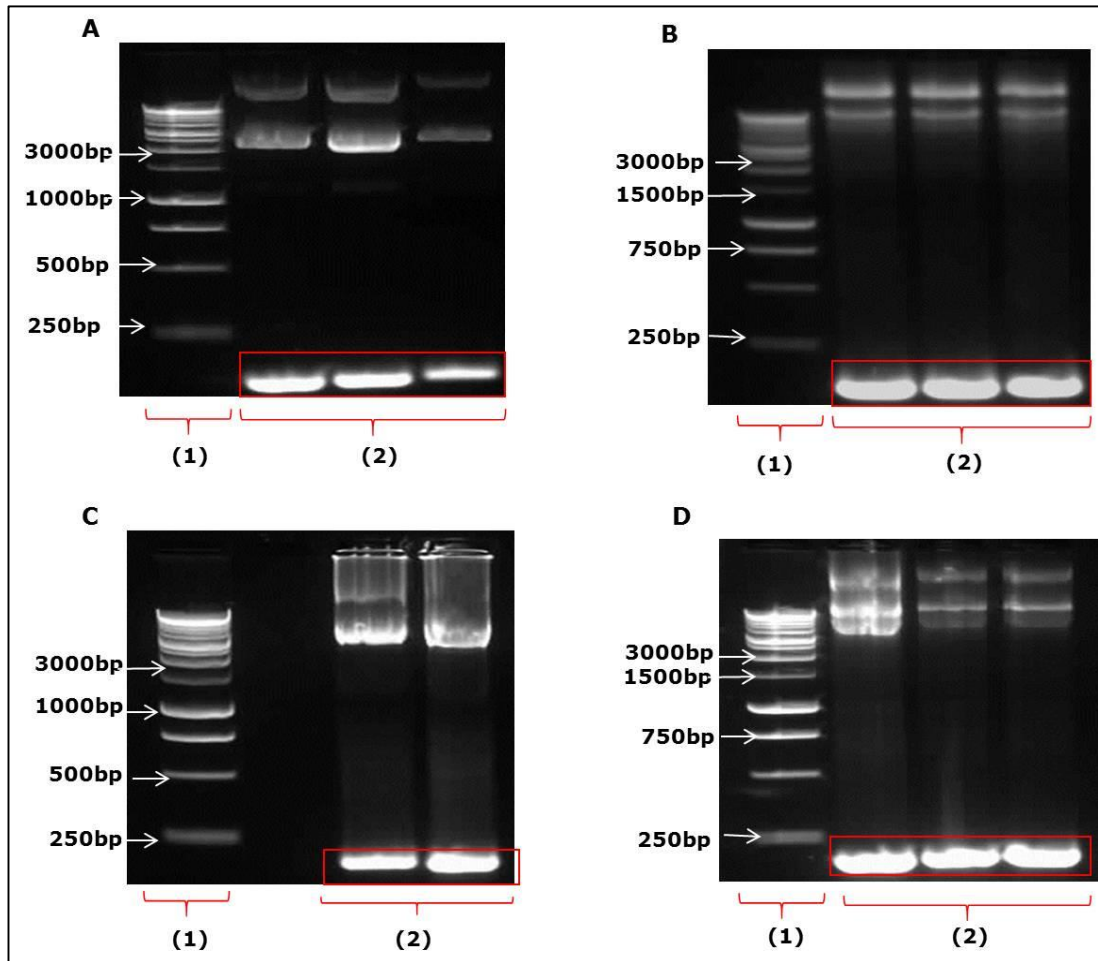


Figure 3.2-1 Confirmation of the presence of *Leishmania* genes in bulked DNA constructs by PCR

(A and B) the *L. mexicana* GP63 gene was amplified from VR1012-*L.mexgp63* and pcDNA3.1/Hygro(-)-*L.mexgp63* plasmids, respectively, using pair 1 primers (Table 2.2.2), lane 1 10kb DNA ladder, lane 2 amplified *L. mexicana* GP63 gene (200bp). (C) 200bp PCR product of *L. donovani* centrin1 from pCR®II-TOPO®-Ldcen1 plasmid (lane 2) using pair 5 primers (Table 2.2.2). (D) PCR amplification of *L. donovani* centrin3 gene (200bp) in pcDNA3.1/Hygro(-)-Ldcen3 plasmid (lane 2) using pair 3 primers (Table 2.2.2).

3.2.2 Construction of pcRT7/CT-TOPO-L.mexgp63 plasmid

Due to the lack of matched restriction enzymes that can cut the full length of the *L. mexicana* GP63 gene, and correspondingly digest the target pcRT7/CT-TOPO plasmid, new primers (Pair 2, table 2.2.2), were designed in order to amplify the target gene by PCR. Forward and reverse primers containing restriction sites for Hind III and Xba I restriction enzymes, respectively were designed. The new primers were successfully amplified 1940pb of *L. mexicana* GP63 gene by PCR (Figure 3.2.2.A). Two enzymes Hind III and Xba I were used to digest the amplified *L. mexicana* GP63 gene and pCRT7/CT-TOPO (Plasmid map figure 2.2.2), in order to create the sticky complementary ends (Figure 3.2.2.B). On completion of the cloning process, three methods were used to prove the presence of the newly cloned *L. mexicana* GP63 gene in the pcRT7/CT-TOPO plasmid:

Firstly, digestion and restriction of the newly constructed pcRT7/CT-TOPO-L.mexgp63 DNA construct using Hind III and Xba I restriction enzymes (Figure 3.2.2.C).

Secondly, the full length of *L. mexicana* GP63 was amplified by PCR using the newly prepared DNA construct as DNA template (Figure 3.2.2.D).

Thirdly, the newly cloned gene was confirmed by sequencing (Source BioScience, UK). Due to the length of *L. mexicana* GP63 gene, three sets of primers were used to fully sequence the target gene. The result shows that no miss-match occurred during the sub-cloning process of the new pcRT7/CT-TOPO-L.mexgp63 DNA construct (Appendix 1).

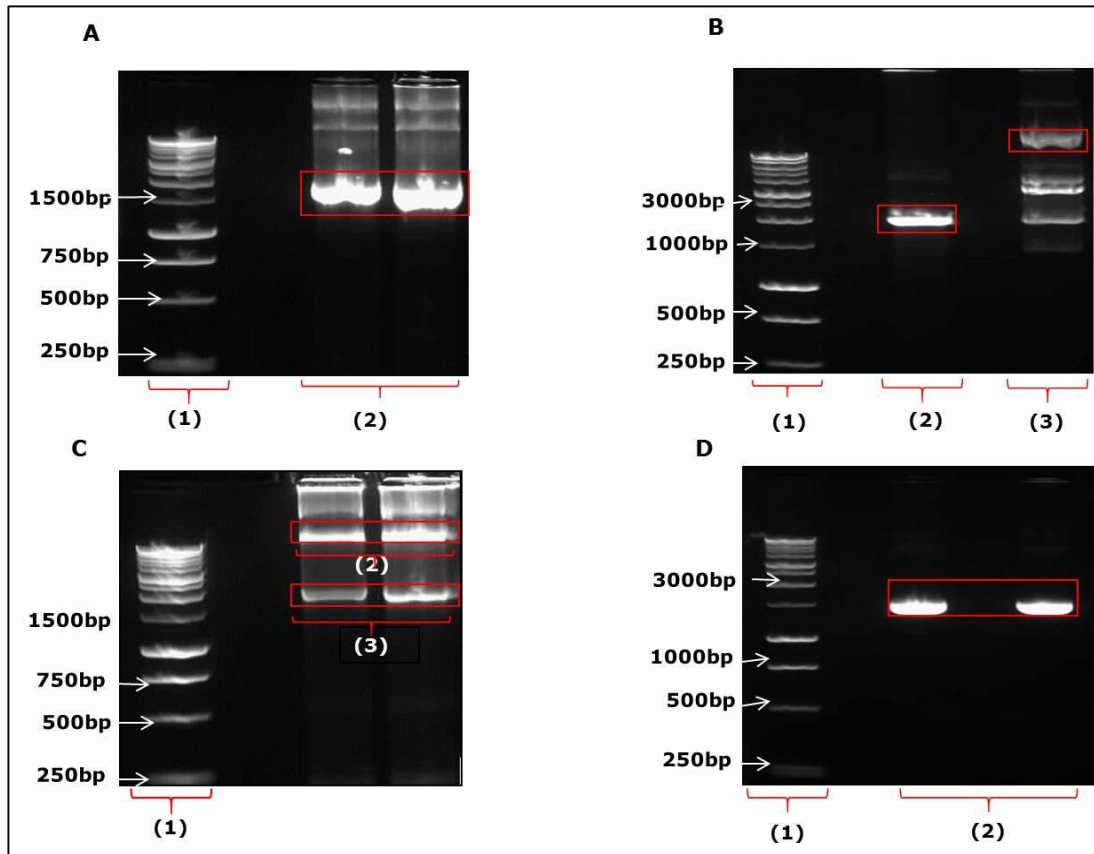


Figure 3.2-2 Construction of pcRT7/CT-TOPO-L.mexgp63 plasmid

(A) The full length of *L. mexicana* GP63 gene was amplified by PCR from VR1012-L.mexgp63 DNA plasmid using pair 2 primers (Table 2.2.2). Lane 1 1kp DNA ladder, lane 2 amplified *L. mexicana* GP63 gene (1494bp). (B) Digestion and restriction of the PCR product (*L. mexicana* GP63 gene, lane 2, 1494bp), and pcRT7/CT-TOPO plasmid (lane 3, 2700bp), with Hind III and Xba I restriction enzymes, linear form of the digested plasmid was gel extracted. (C) The confirmation of the presence of *L. mexicana* GP63 gene in newly prepared pcRT7/CT-TOPO-L.mexgp63 construct using the restriction enzymes method, pcrT7/CT-TOPO-L.mexgp63 was digested by Hind III and Xba I restrictions enzymes. Lane 1 1kp DNA ladder, band 2 digested pcRT7TOPO-L.mexgp63 plasmid, band 3 *L. mexicana* GP63 gene (1494bp). (D) Confirmation of the presence of *L. mexicana* GP63 gene in newly prepared pcRT7/CT-TOPO-L.mexgp63 by PCR (1494bp), the complete *L. mexicana* GP63 gene was amplified from pcRT7/CT-TOPO-L.mexgp63 plasmid (band 2) using pair 2 primers (Table 2.2.2).

3.2.3 Construction of pcRT7/CT-TOPO-Ldcen1 plasmid

L. donovani centrin1 was initially cloned into pCR®II-TOPO®-Ldcen1 plasmid. In order to cut off Ldcen1 from pCR®II-TOPO® and prepare empty pcRT7/CT-TOPO (plasmid map figure 2.2.3 and 2.2.2) respectively, both DNA constructs were digested with the same restriction enzymes (Hind III and Xba I) to create sticky complementary ends (Figure 3.2.3.A). After gel purification, both the empty pcRT7/CT-TOPO plasmid and *L. donovani* centrin1 gene were ligated and transformed into *E. coli* complement cells as previously described in sections 2.2.1.2.1 and 2.2.1.2.2. The cloning of the *L. donovani* centrin1 gene into pcRT7/CT-TOPO plasmid was confirmed by PCR and sequencing (Figure 3.2.3.B and Appendix 2).

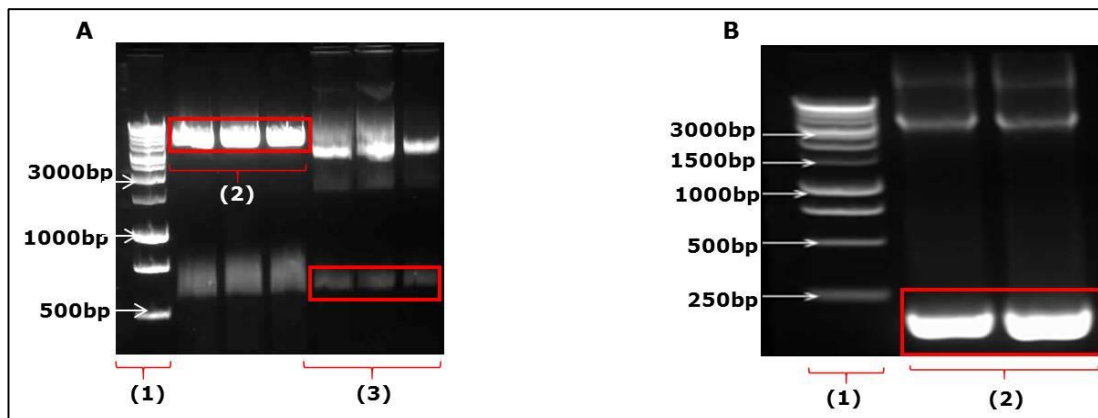


Figure 3.2-3 Construction of pcRT7/CT-TOPO-Ldcen1 plasmid

(A) pcT7/CT-TOPO-Ldcen3 and pCR® II-TOPO® -Ldcen1 plasmids were digested with Hind III and Xba I restriction enzymes. Lane 1 11kbp DNA ladder and band 2 represent empty pcRT7/CT-TOPO plasmid (2700bp). Band 3 represent excised *L. donovani* centrin1 gene from pCR®II-TOPO®-Ldcen1 plasmid (500bp). (B) Confirmation of the presence of *L. donovani* centrin1 gene in newly prepared pcRT7/CT-TOPO-Ldcen1 DNA construct by PCR. The 200bp PCR product (Lane 2) was amplified using pair 3 primers (Table 2.2.2).

3.2.4 Construction of VR1012-Ldcen1 plasmid

VR1012-L.mexgp63 DNA construct was first digested with EcoR I restriction enzyme to remove the *L. mexicana* GP63 gene. *L. donovani* centrin1 was also cut off from the pCR®II-TOPO®-Ldcen1 DNA construct using the same restriction enzyme (Figure 3.2.4.A). Both the gene of interest and the target plasmid were subjected to the ligation step followed by transformation, as described in sections 2.2.2.1.4, 2.2.1.2.1, and 2.2.1.2.2. The newly cloned *L. donovani* centrin1 gene into VR1012 plasmid was confirmed by PCR (Figure 3.2.4.B) and sequencing.

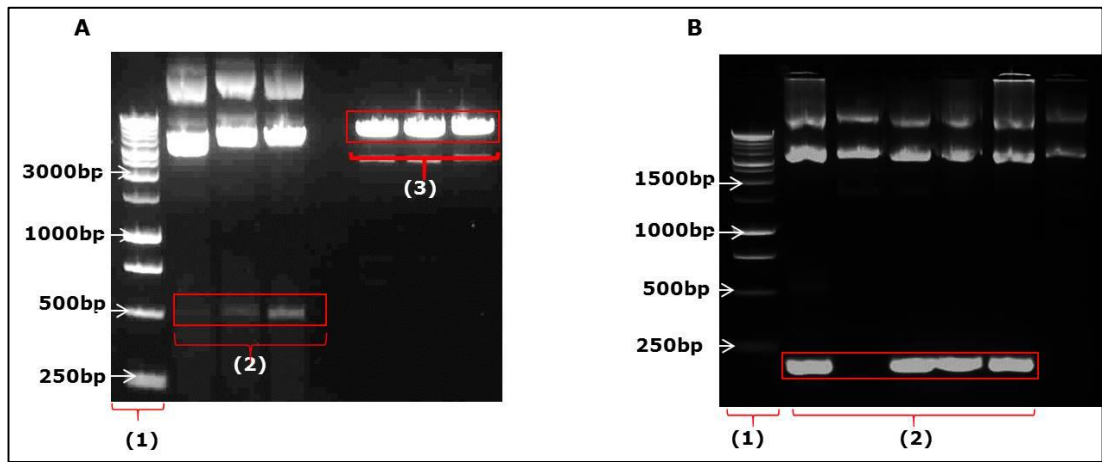


Figure 3.2-4 Construction of VR1012-Ldcen1 plasmid

(A) Agarose gel electrophoresis illustrates the digestion of VR1012-L.mexgp63 and pCR®II-TOPO® Ldcen1 with EcoR I restriction enzyme. Lane 1 1kp DNA ladder, band 2 represent excised *L. donovani* centrin1 gene (500bp). Lane 3 represent the empty VR1012 plasmid (6600bp). (B) Confirmation of the presence of *L. donovani* centrin1 gene in newly prepared VR1012-Ldcen1 DNA construct by PCR, a 200bp *L. donovani* centrin1 PCR produce (Lane 2) was amplified using pair 5 primers (Table 2.2.2).

3.2.5 Construction of VR1012-Ldcen3 plasmid

Due to the lack of required restriction enzyme sites, a set of new primers which have the sequence of Sall and BgIII enzymes forward and reverse primer, respectively was designed (Table 2.2.2). The new primers were used to amplify a full length of *L. donovani* centrin3 gene by PCR (Figure 3.2.5.A). The fully amplified PCR product (*L. donovani* centrin3) and VR1012 plasmid were digested with Sall and BgIII restriction enzymes to create the complementary sticky ends. After gel purification, VR1012 plasmid and *L. donovani* centrin3 gene were ligated at a ratio of 1:3 (plasmid: insert). The new product was transformed into *E. coli* complement cells to produce more copies. The new VR1012-Ldcen 3 DNA construct was subjected to a further step to confirm the presence of the *L. donovani* centrin3 gene (Figures 3.2.5.B, and Appendix 3).

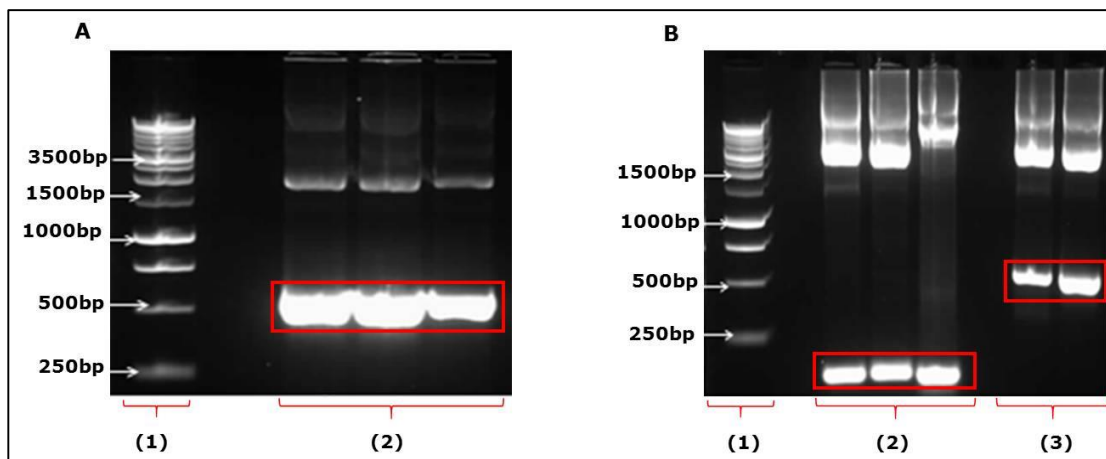


Figure 3.2-5 Construction of VR1012-Ldcen3 plasmid

(A) The full-length *L. donovani* centrin3 was amplified from the pcRT7/CT-Topo-Lcen3 plasmid by PCR using pair 4 primers (Table 2.2.2). Lane 1 1kp DNA ladder, Lane 2 represent amplified *L. donovani* centrin3 gene (500bp). (B) Confirmation of the presence of newly cloned *L. donovani* centrin3 gene into VR1012 plasmid. Two sets of primers (Pair 2 and 3 in Table 2.2.2) were used to amplify 200bp (2) and 500bp (3), respectively. Lane1 1kp DNA ladder, lane 2 and 3 represent the 200bp and 500bp *L. donovani* centrin3 gene PCR product, respectively.

3.2.6 Construction of pcDNA3.1/Hygro(-)-Ldcen1 plasmid

The pCR®II-TOPO®-Ldcen1 plasmid was digested with Hind III and Xba I restriction enzymes (see the plasmid map figures 2.2.2 and 2.2.4), to remove *L. donovani* centrin1, and pcDNA3.1/Hygro(-) plasmid was also digested with Hind III and Xba I restriction enzymes (Figure 3. 2. 6. A). The target plasmid (pcDNA3.1/Hygro(-)) and *L. donovani* centrin1 gene were ligated at a ratio of 1:3 (plasmid: insert). The new DNA construct was bulked up by *E. coli* transformation, and cloning success was confirmed by digestion and restriction (Figure 3.2.6. B), PCR, and sequencing.

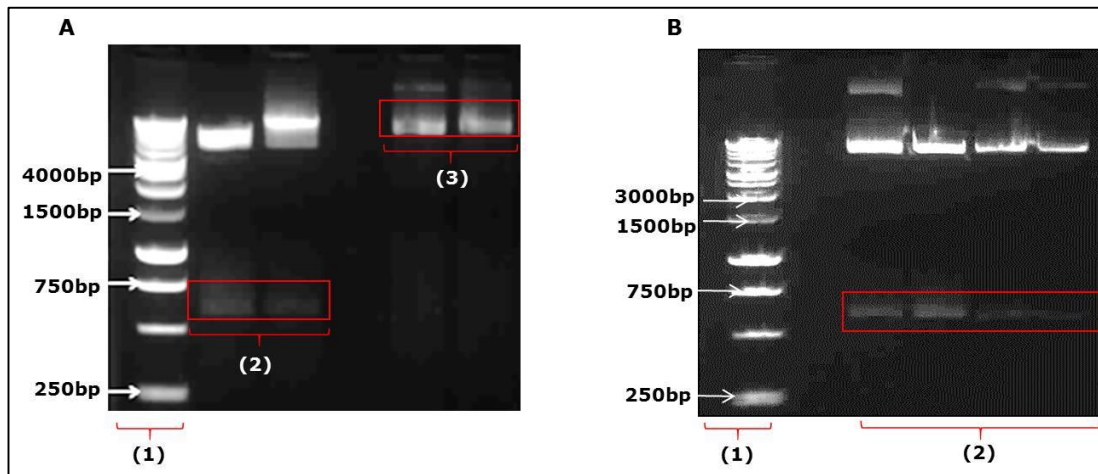


Figure 3.2-6 Construction of pcDNA3.1/Hygro(-)-Ldcen1 plasmid

(A) Digestion of pCR®II-TOPO®-Ldcen1 and empty pcDNA3.1/Hygro(-) plasmids with Hind III and Xba I restriction enzymes. Lane 1 kp DNA ladder, lane 2 is the digested *L. donovani* centrin1 gene (500bp), and lane 3 represent the digested pcDNA3.1/Hygro(-) plasmid (5600bp). (B) Confirmation of the presences of Ldcen1 in newly prepared pcDNA3.1/Hygro(-)-Ldcen1 DNA construct by restriction and digestion. The newly prepared pcDNA3.1/Hygro(-)-Ldcen1 DNA construct was digested with Hind III and Xba I restriction enzymes. Lane1 1kp DNA ladder, lane 2 represents the digested *L. donovani* centrin1 gene (500bp) from pcDNA3.1/Hygro(-)-Ldcen1 plasmid.

3.2.7 Transfection of MCF-7 tumour cells

To investigate whether the constructed plasmids were able to express *Leishmania* genes in mammalian cells, MCF-7 tumour cells were transfected with the prepared DNA constructs encoding *Leishmania* genes using Lipofectamine 2000, according to the manufacturer's recommended protocol (details are in the Methods section, 2.2.3). After three passages in the selective medium, mRNA was extracted from the transfected cells and converted into cDNA. The presence of the *L. donovani* centrin1 and *L. donovani* centrin3 genes in the transfected cells was determined by qPCR analysis. The qPCR results clearly show the presence of a significant amount of RNA message for *L. donovani* centin1 and *L. donovani* centin3 induced by 3 different plasmids, respectively Figure 3.2.7. A. B. The expression of *L. mexicana* GP63 protein in transfected cells was also determined by immunofluorescence antibody staining. Images presented in (Figure 3.2.7.C) clearly show the expression of GP63 protein (white arrows), whereas no GP63 protein was detected in the control cells.

3) *Leishmania* DNA constructs immunogenicity

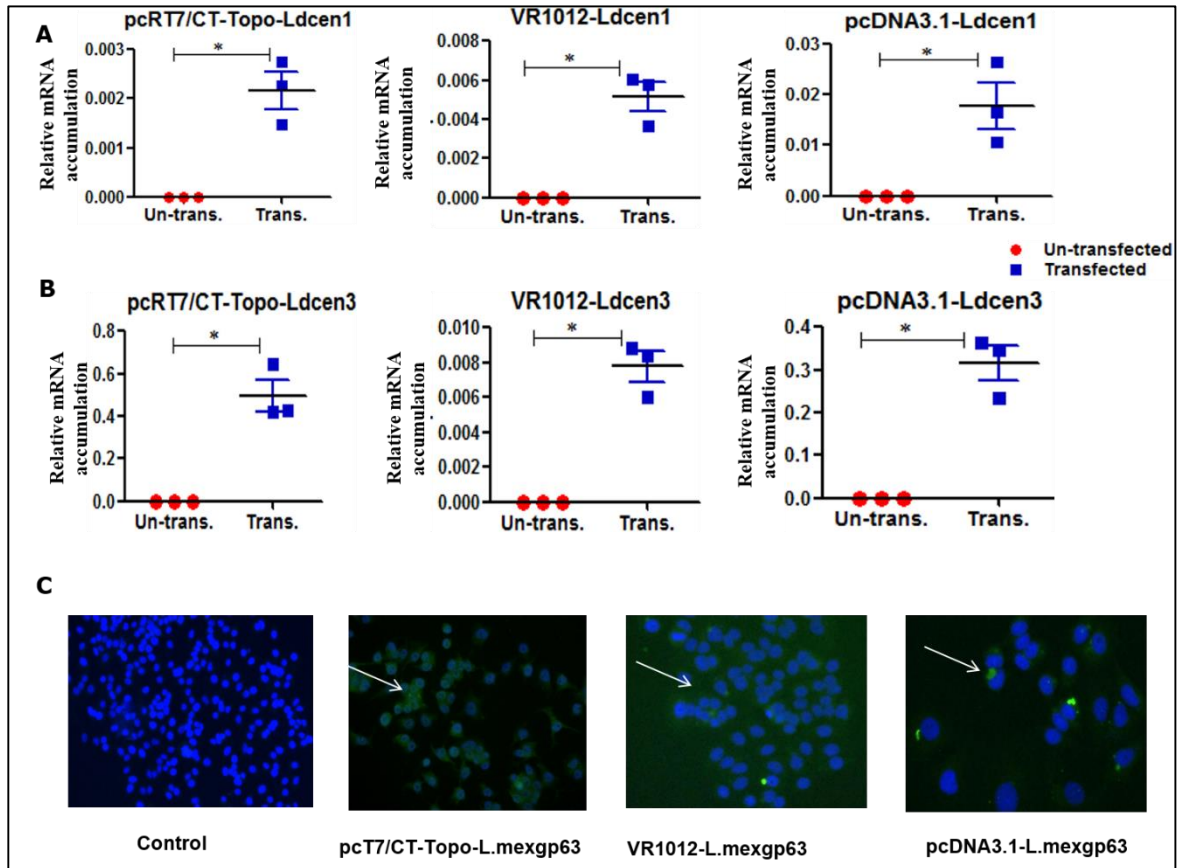


Figure 3.2-7 Transfection of MCF-7 cell line with prepared DNA constructs

MCF-7 tumour cells were transfected with DNA constructs encoding *L. donovani* centrin1 gene (Panel A), or DNA constructs encoding *L. donovani* centrin3 gene (Panel B) using Lipofectamine™ 2000. Graphs clearly showed that both genes were significantly expressed in transfected compared to un-transfected cells. The results represent the average of three independent experiments. Data are presented as mean + SEM.. Panel (C) shows the immunofluorescence staining using anti *L. mexicana* GP63 protein in the transfected MCF-7 cells. The images clearly show the expression of GP63 protein (arrowed) detected by incubating the transfected cells with primary anti-*L. major* GP63 antibody, followed by incubation with goat anti-mouse IgG2a: FITC. The experiment was repeated twice.

3.3 Immunogenicity of *Leishmania* DNA constructs

As has been mentioned in the Methods section, different tissues were collected from mice after gene gun immunisation with *Leishmania* DNA constructs, and were subjected to various immune assays to assess immune responses as described below:

3.3.1 IFN γ Elispot assay

The IFN- γ secretion as result of *L. mexicana* SLA stimulation, after the immunisation with *Leishmania* DNA constructs was checked using an IFN γ Elispot assay. Whole splenocytes population were collected two weeks after the last immunisation. Splenocytes were seeded at a concentration of 1×10^6 cells per well in Elispot plates and stimulated with 10 μ g per ml SLA. Plates were incubated at 37°C in 5% v/v CO₂ for 48 hours. Splenocytes were then re-stimulated with 30 and 50 μ g per ml SLA and further incubated for 24 hours. The results, shown in Figure 3.3.1 A, B, C, clearly show that immunisation with the *Leishmania* DNA constructs induced significant IFN- γ secretion in response to stimulation of splenocytes with *L. mexicana* SLA, which was dose-dependent since using a high dose of SLA in the second stimulation caused a significant increase in IFN- γ secretion by stimulated cells.

The results in Figure 3.3.1.D show that no significant secretion of IFN- γ was detected in mice immunised with empty plasmids. However, a slight increase in the IFN- γ secretion of splenocytes from mice immunised with empty plasmids was observed.

The IFN γ Elispot results show that using different plasmids encoding the same gene did not significantly alter the immunogenicity of the DNA constructs. However, the immunogenicity was affected by the *Leishmania* gene type. *L. donovani* centrin3 cloned in the three different plasmids induce more IFN- γ secretion.

3) *Leishmania* DNA constructs immunogenicity

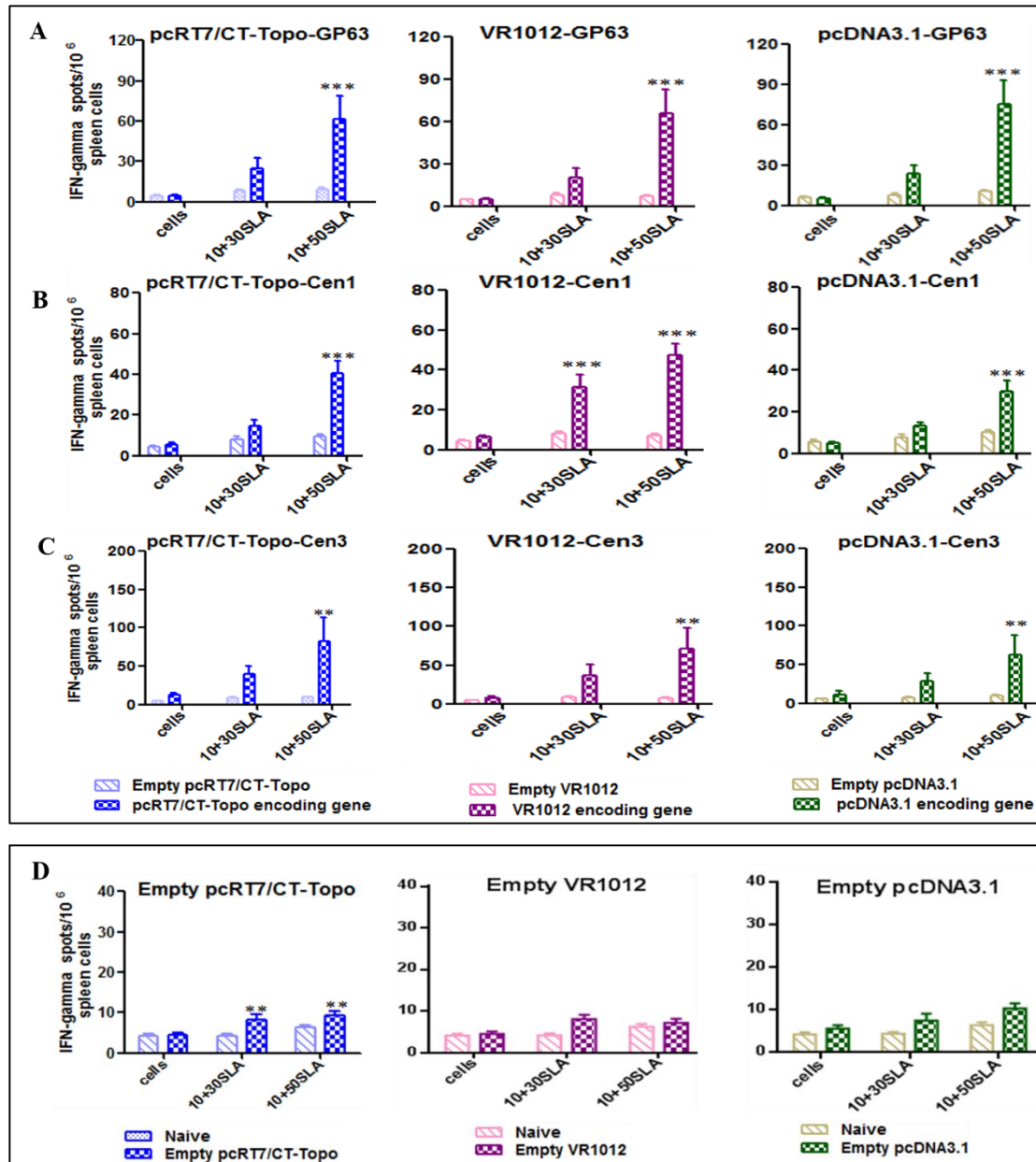


Figure 3.3-1 IFN- γ secretion determined by Elispot Assay in splenocytes of mice immunised with DNA constructs following *in vitro* stimulation with *L. mexicana* SLA

Splenocytes from immunised mice with plasmids encoding *L. mexicana* GP63 gene (A), *L. donovani* centrin1 gene (B), *L. donovani* centrin3 gene (C) and control mice (D) were cultured at a concentration of 10^6 cells per well in coated Elispot plate, and stimulated with $10\mu\text{g}$ per ml SLA. 48 hours later, splenocytes were stimulated again with 30 and $50\mu\text{g}$ per ml SLA for 24 hours. The plate was then developed and number of spots counted by an Elispot reader. The test was performed in triplicate for each SLA concentration, and the negative control (cells) was left without antigen (SLA). Two controls were used: splenocytes from mice immunised with empty plasmids or immunised with empty bullets. The SEB positive control induced 200 spots. Data are represented the average of three independent experiments for each DNA construct. ** $P < 0.01$, *** $P < 0.001$ (Two way ANOVA/GgraphPad Prism). The comparison was performed between plasmids encoding *Leishmania* genes and empty plasmids. Data are presented as mean + SEM.

3.3.2 Proliferation of splenocytes in response to SLA stimulation

Balb/c mice were immunised by gene gun three times with DNA constructs as described in the materials and methods section (2.2.4.3.2). Two weeks after the last immunisation, the mice were sacrificed and the splenocytes plated into 96 well plates at a concentration of 5×10^5 per well in a total volume of 200 μ l of T cell medium and stimulated with 10 μ g SLA on the day of harvesting (day zero). After 72 hours, splenocytes were re-stimulated using two doses (30 and 50 μ g per ml) SLA. Cell proliferation was estimated by thymidine uptake assay. Optimization experiments illustrated that the optimum T cell proliferation required a double *in vitro* stimulation with SLA (10 μ l per ml on day zero, followed by 30 or 50 μ l per ml on day three).

Results in (Figure 3.3.2.A) illustrate the proliferation of the splenocytes from mice immunised with the three plasmids encoding the *L. mexicana* GP63 gene. Generally, the proliferation of splenocytes from mice immunised with plasmids encoding this gene was significantly higher, when compared to mice immunised with empty plasmids as a control. Moreover, although the proliferation of immunised mice splenocytes was significant using 10+30 μ g SLA compared to the control splenocytes, the stimulation with 10+50 μ g SLA induced more proliferation. The graph also shows that the highest proliferation rate was obtained in mice immunised by pcRT7/CT-TOPO-*L.mexgp63*, followed by pcDNA3.1/Hygro(-)-*L.mexgp63* plasmid.

Figure 3.3.2.B shows the proliferation of splenocytes in mice immunised with the three plasmids encoding *L. donovani* centrin1. Similarly, the proliferation of splenocytes from plasmids encoding centrin1 immunised mice was high following *in vitro* stimulation with 10+50 μ g SLA compared to mice immunised with empty plasmids. The results also indicate that immunisation with the VR1012-Ldcen1 plasmid resulted in a higher proliferation rate compared to immunisation with the pcRT7/CT-TOPO-Ldcen3 and pcDNA3.1/Hygro(-)-Ldcen3 constructs. Unlike immunisation with plasmids encoding *L. mexicana* GP63 and *L. donovani* centrin1, there was no significant difference between the proliferation of splenocytes from immunised mice with pcRT7/CT-TOPO-Ldcen3 and pcDNA3.1/Hygro(-)-Ldcen3 constructs and mice immunised with empty plasmids (Figure 3.3.2.C). However, there was a significant difference in splenocytes proliferation from mice immunised with VR1012-Ldcen3 compared to control cells (splenocyte from mice immunised with empty VR1012 plasmid).

3) Leishmania DNA constructs immunogenicity

Results in Figure 3.3.2.D, show there was no difference in the proliferation of splenocytes harvested from immunised mice with empty plasmids and empty bullets.

3) *Leishmania* DNA constructs immunogenicity

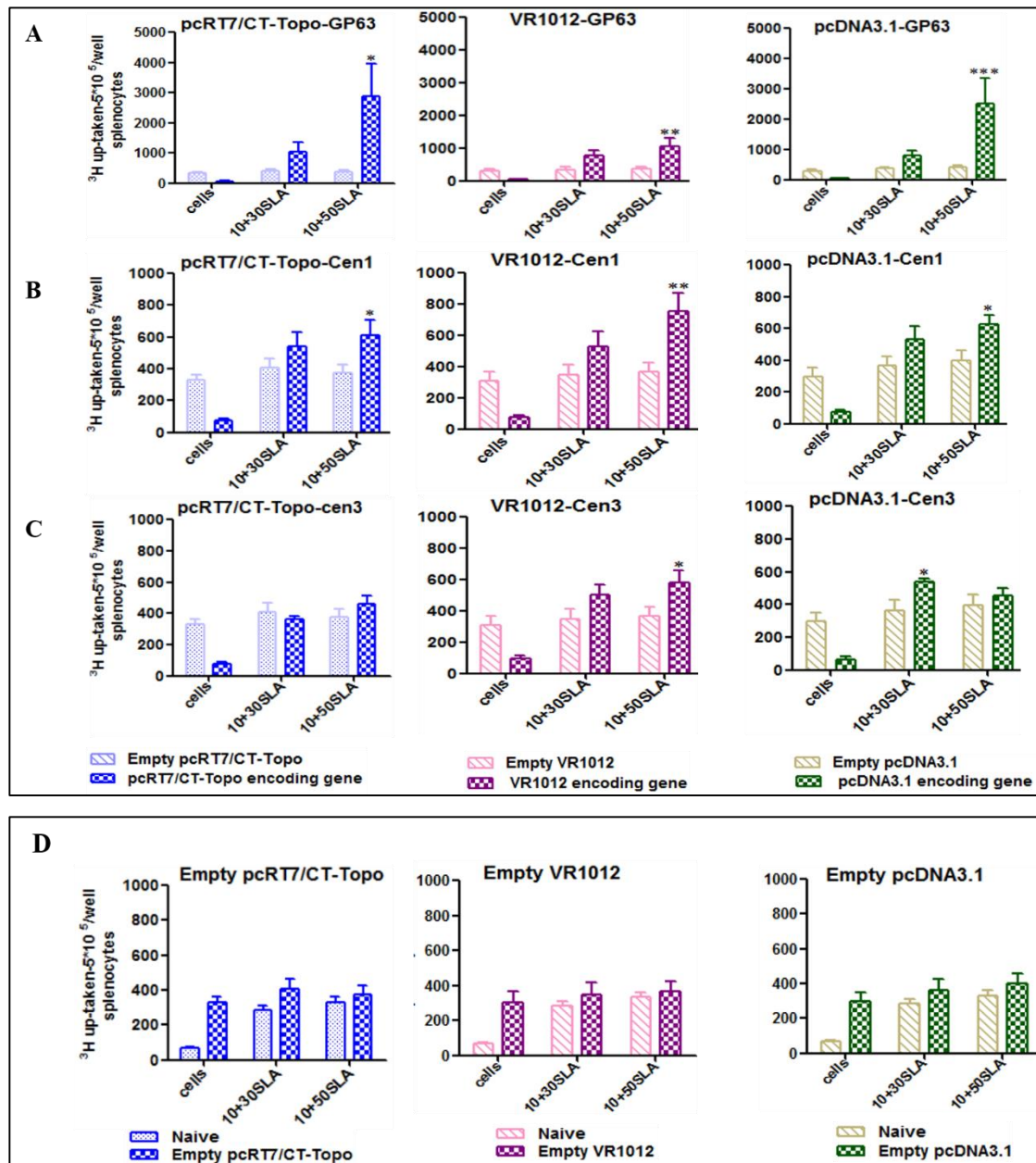


Figure 3.3-2 Proliferation of splenocytes stimulated with SLA

The immunogenicity of DNA constructs was estimated by thymidin up-take proliferation assay. On day zero, splenocytes from mice immunised with plasmids encoding *L. mexicana* GP63 (A), *L. donovani* centrin1 (B), and *L. donovani* centrin3 (C) genes, were cultured at a concentration of 5×10^5 cells per well in T cell medium using 96 well plates. Cells were first stimulated with $10 \mu\text{g}$ per ml SLA for 72 hours, then re-stimulated with 30 and $50 \mu\text{g}$ per ml SLA, and plates were further incubated for 72 hours and labelled with $1 \mu\text{l}$ Ci [^3H]-Thymidine per well. Plates were harvested and counted. (D) Proliferation of splenocytes from mice immunised with empty plasmids or empty bullets. The results represented the average of three independent experiments for each DNA constructs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Two way ANOVA/GraphPad Prism). The comparison was performed between plasmids encoding *Leishmania* genes and empty plasmids. Data are presented as mean + SEM.

3.3.3 Proliferation of lymph node lymphocytes in response to SLA stimulation

Ten lymph nodes from each mouse were collected on the day of harvesting (Appendix 4), and lymphocytes were flushed out using serum-free medium. Cells were cultured and treated as described for splenocytes. Interestingly, a similar pattern of results to that of splenocyte proliferation was observed. Results illustrated that there was not much difference in the proliferation of lymphocytes from mice that had been immunised with the three plasmids encoding the *L. mexicana* GP63 gene (Figure 3.3.3. A). Results of Figure 3.3.3. B shows the proliferation of lymphocytes from mice immunised with three plasmids encoding *L. donovani* centrin1. The pcDNA3.1/Hygro(-)-Ldcen1 construct resulted in the highest proliferation rate at the two tested SLA doses, while the lower proliferation rate was obtained in mice immunised with pcRT7/CT-TOPO-Ldcen1. However, the proliferation of lymphocytes from immunised mice was still higher than that of lymphocytes from mice which had been immunised with empty plasmids. Figure (3.3.3.C) shows that immunisation with VR1012-Ldcen3 and pcDNA3.1/Hygro(-)-Ldcen3 induced a significant proliferation compared to that of empty plasmids following stimulation with 10+30 and 10+50µg SLA per ml. Although immunisation with pcR7/CT-TOPO-Ldcen3 induced high proliferation, statistical analysis showed no significant differences when compared to the control (immunised with empty plasmids). Two controls (immunised with empty plasmids and empty bullet) were also included in these experiments, and the results clearly show a slight increase in the lymphocyte proliferation of mice immunised with empty plasmids compared to empty bullets. However, the statistical analysis showed there were no significant differences (Figure 3.3.3.D).

3) *Leishmania* DNA constructs immunogenicity

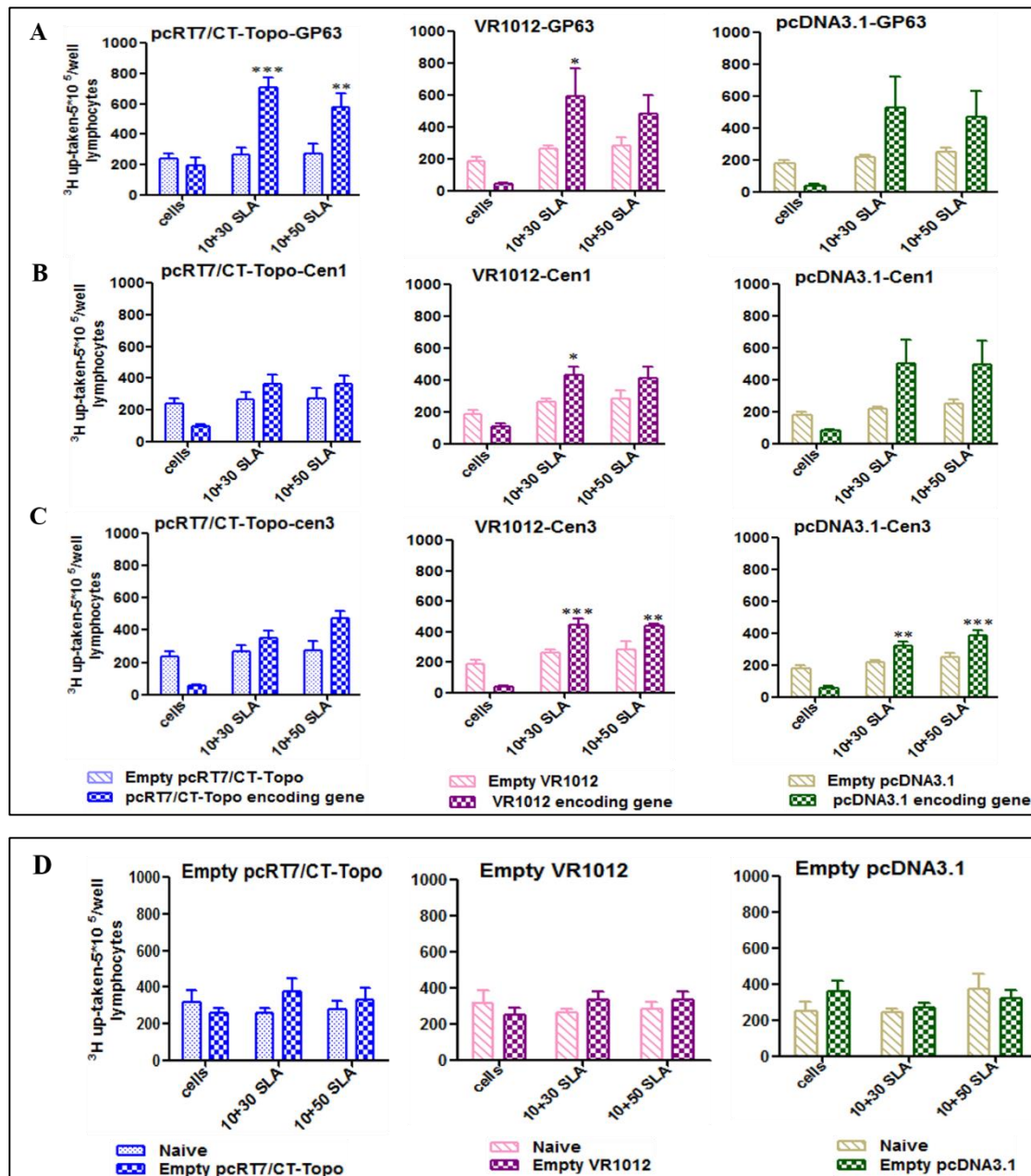


Figure 3.3-3 Proliferation of lymph node lymphocytes stimulated with SLA

On day zero, lymph node lymphocytes from mice immunised with plasmids encoding *L. mexicana* GP63 gene (A), *L. donovani* centrin1 (B), and *L. donovani* centrin3 gene (C) genes were cultured at a concentration of 5×10^5 cells per well in T cell medium using a 96 well plates. Cells were first stimulated with $10 \mu\text{g}$ per ml SLA for 72 hours, re-stimulated with 30 or 50 $10 \mu\text{g}$ per ml SLA, and further incubated for 72 hours with $1 \mu\text{l}$ Ci [^3H]-Thymidine per well. Cells were then harvested and counted. Two control mice immunised with empty plasmids and empty bullets were also included (D). The results represent the average of three independent experiments for each DNA construct. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Two way ANOVA/GraphPad Prism). The comparison was preformed between plasmids encoding *Leishmania* genes and empty plasmids. Data are presented as mean + SEM.

3.3.4 The CD4 and CD8 profile in immunised mice.

In this study, the effect of immunisation with the different *Leishmania* DNA constructs on the percentage of CD4⁺ and CD8⁺ T cells in spleens and lymph nodes was investigated using flow cytometry. Cells from spleens and lymph nodes were harvested two weeks after the last immunisation, and cell suspensions prepared as described in sections 2.2.4.4 and 2.2.4.5, were stained with anti-mouse CD4⁺ and CD8⁺ antibodies and analysed by flow cytometry. The results illustrate that immunisation has caused an alteration in the percentage of CD4⁺ and CD8⁺ cells depending on the source of T cells and the encoding gene. In general, more variations in CD4⁺ and CD8⁺ cell profiles were observed in spleens compared with lymph nodes. Results in Figure 3.3.4. A, show that immunisation with three different plasmids encoding *L. mexicana* GP63 caused a significant increase in the percentage of the CD4⁺ population. Similar results were obtained using pcRT7/CT-TOPO and VR1012 plasmids encoding *L. donovani* centrin1. Figure 3.3.4. B illustrates that the CD8⁺ cell percentage was also increased, after the immunisation, but not significantly. This was associated more with the use of pcRT7/CT-TOPO plasmid encoding the three *Leishmania* genes, compared with VR1012 and pcDNA3.1/Hygro(-) plasmids.

Similarly, in the lymph nodes, immunisation with the plasmids encoding *L. mexicana* gp63 triggered the most significant increase in the CD8⁺ population, but a significant increase was only observed after immunisation with pcRT7/CT-TOPO plasmid encoding *L. donovani* centrin1 (Figure 3.3.4.C). Interestingly, the results in Figure 3.2.4.D, show that there are no differences in the percentage of CD8⁺ in control and immunised mice.

3) *Leishmania* DNA constructs immunogenicity

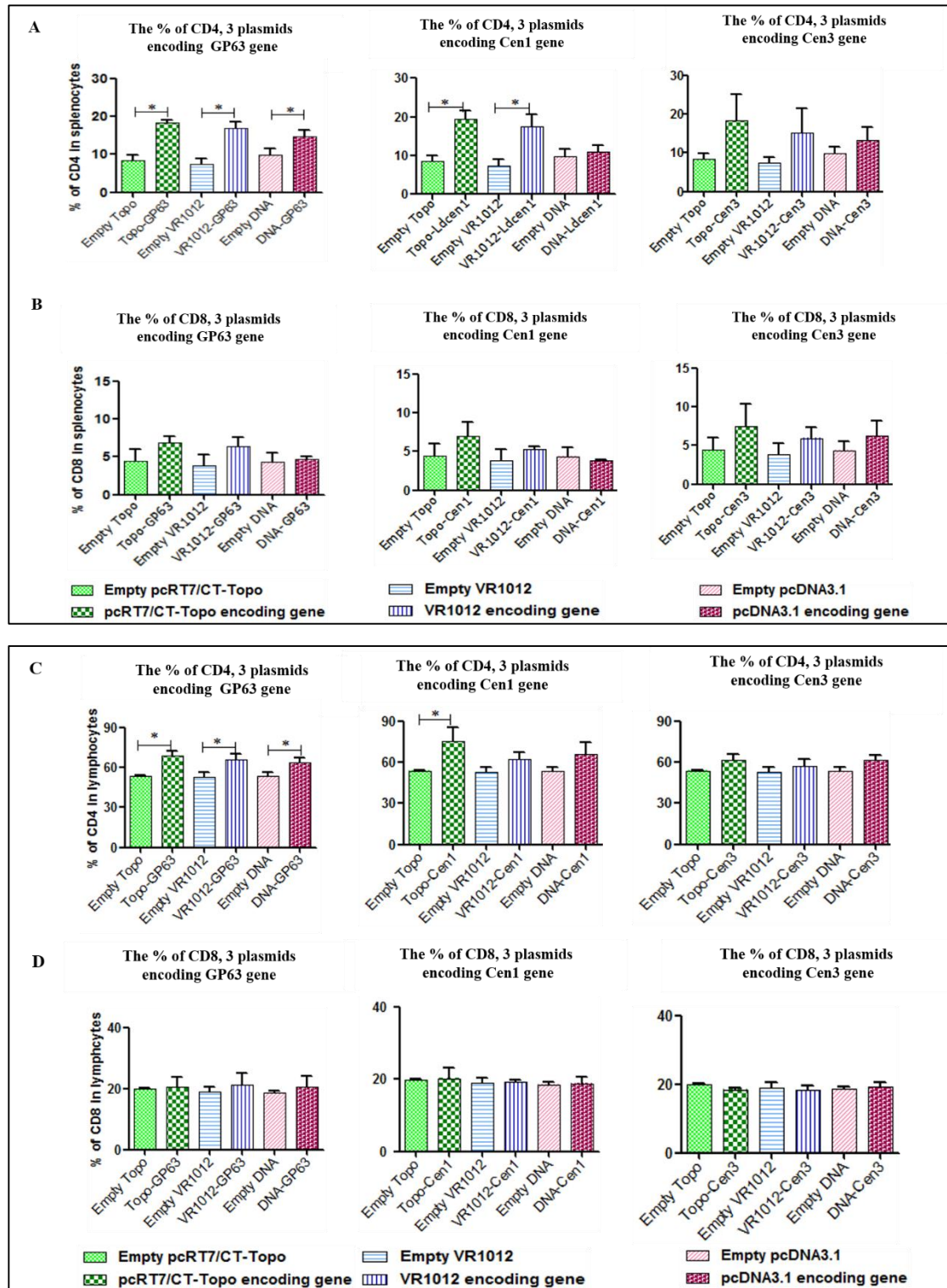


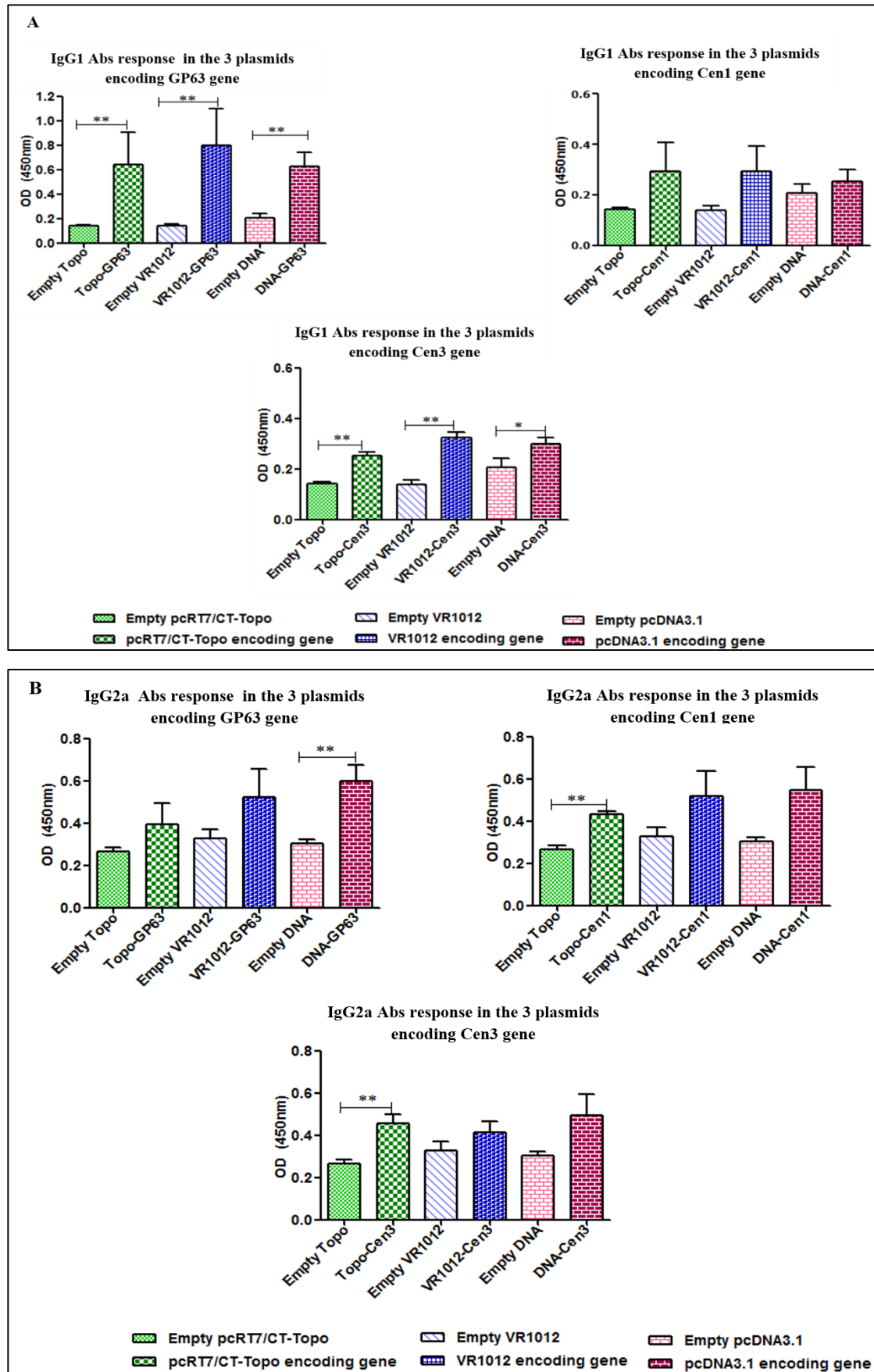
Figure 3.3-4 Profiles of CD4 and CD8 in spleens and lymph nodes of immunised and control mice by flow cytometry analysis

On the day of harvesting, splenocytes (A, B) and lymph node cells (C, D) were stained using FITC anti-mouse CD4⁺ and RPE anti-mouse CD8⁺ according to the manufacturer's protocol. The data represent the percentage of CD4 and CD8 in splenocyte populations of three independent experiments for each DNA construct. *P<0.05 using the Mann-Whitney U test. The comparison was performed between plasmids encoding *Leishmania* genes and empty plasmids. Data are presented as mean + SEM..

3.3.5 Antibody response in immunised mice

To estimate the level of total IgG, IgG1 and IgG2a isotypes following immunisation with the different *Leishmania* DNA constructs, blood samples of immunised and control Balb/c mice were collected two weeks after the last immunisation (the day of harvesting), and subjected to ELISA analysis (section 2.2.4.10). The results clearly show an overall increase in the level of IgG1 in mice immunised with the plasmids encoding *Leishmania* genes compared to those immunised with empty plasmids (controls). Figure 3.3.5.A, shows that the immunisation of Balb/c mice with the three plasmids encoding *L. mexicana* GP63 and *L. donovani* centrin3 genes induced high levels of IgG1 isotype compared to control mice. Figure (3.3.5.B) shows that the levels of IgG2a were also high in the serum of immunised mice compared to control groups. pcDNA3.1/Hygro(-)-L.mexgp63 was the only construct that induced a significant increase in IgG2a isotype, compared to pcRT7/CT-TOPO-L.mexgp63 and VR1012-L.mexgp63. The results also show that pcRT7/CT-TOPO encoding either *L. donovani* centrin1 gene or *L. donovani* centrin3 gene has induced a significant increase in IgG2a isotype, compared to other constructs encoding the same genes. The results illustrated no differences in levels of the total IgG isotype following immunisation with the different DNA constructs encoding *Leishmania* genes and mice immunised with empty plasmids (Figure 3.3.5.C).

3) *Leishmania* DNA constructs immunogenicity



3) *Leishmania* DNA constructs immunogenicity

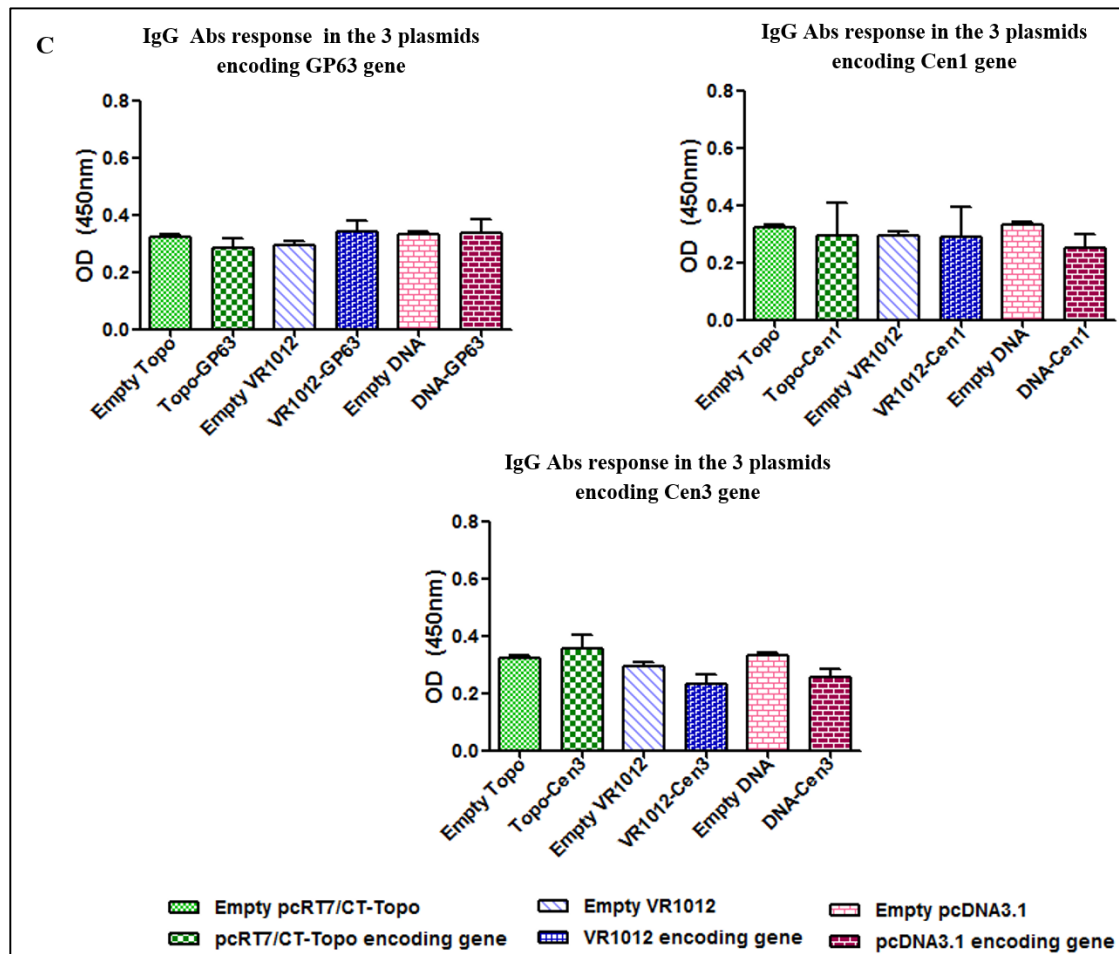


Figure 3.3-5 Antibody responses in the sera of immunised mice

Serum was obtained from blood samples on the day of harvesting. Specific anti *Leishmania* antibodies were determined by loading sera from control and immunised mice into plates coated with 5µg SLA per ml, as described in the Materials and Methods section. The results clearly show IgG1 (A) antibody response was higher in mice immunised with the DNA constructs compared to empty plasmids. Anti-*L. mexicana* IgG2 (B) was also high in immunised compared to control mice. However, analysis shows there were no differences in total IgG antibody response between test and control groups (C). The presented data are the average of three independent experiments for each DNA construct. * $P < 0.05$, ** $P < 0.01$ using the Student's t-test. The comparison was performed between plasmids encoding *Leishmania* genes and empty plasmids. Data are presented as mean + SEM.

3.3.6 Survival assay

This assay was developed to assess the effects of immunisation on the ability of macrophages to control the parasite infection. The bone marrow derived macrophages BM-DMs were prepared from the hind limbs of naïve and immunised animals. During 6 days of culture in the presence of mouse GM-CSF, non-adherent cells were gently removed by washing on day 2 and 4. The attached cells were then matured by adding one ml of fresh medium containing LPS at a concentration of 1 µg per ml and incubated at 37°C for 24 hours to induce BM-DMs maturation. Flow cytometry analysis showed that 76.68% of adherent cells expressed F4/80, 3.61% expressed CD11c and 64.26% expressed MHC class I. These results indicate that macrophages can be successfully derived *in vitro* from bone marrow (Figure 3.3.6). To conduct the survival assay, the macrophages from naïve and immunised mice were infected with the late log stage of *L. mexicana* promastigotes for 6 hours then non-attached parasites were removed, and the infected cells were further incubated for 48 hours. Results show that amastigotes can be produced in infected macrophages generated from naïve mice immunised with empty bullets, or empty plasmids as well as macrophages generated from mice immunised with *Leishmania* DNA constructs (Figure 3.3.7. A). However, these amastigotes behaved differently when they were cultured in a 25°C incubator in order to retrieve the promastigote stage. Results show amastigotes isolated from the infected macrophages of immunised mice with plasmids encoding *L. donovani* centrin1 or centrin3 failed to transform into the promastigote stage, compared with empty plasmids (Figure 3.3.7.B, C). Results also show that the amastigotes isolated from macrophages of mice immunised with empty bullets or plasmids successfully produced the promastigote stage (Figure 3.3.7.D). Further analysis was carried out to evaluate whether the concentration of nitrite and IFN-γ have any role on amastigote transformation. Supernatant samples were collected at different time points from macrophages infected with *L. mexicana* promastigotes and subjected to NO and IFN-γ estimation using Griess' reagent and the ELISA method respectively. Results in Figure 3.3.8.A, show that nitrite concentration was significantly lower in macrophages generated from control and mice immunised with plasmids encoding *L. donovani* centrin1 and *L. donovani* centrin3 at 24 and 48 hours respectively following parasite infection. Interestingly, the results of ELISA analysis showed there were no differences in IFN-γ concentration between cells and infected cells supernatant (Figure. 3.3.8. B).

3) *Leishmania* DNA constructs immunogenicity

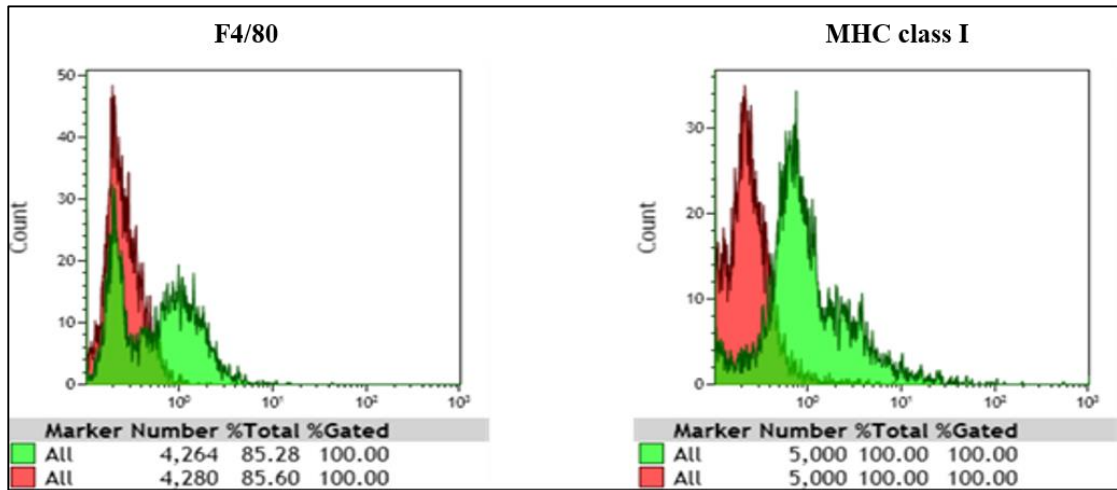
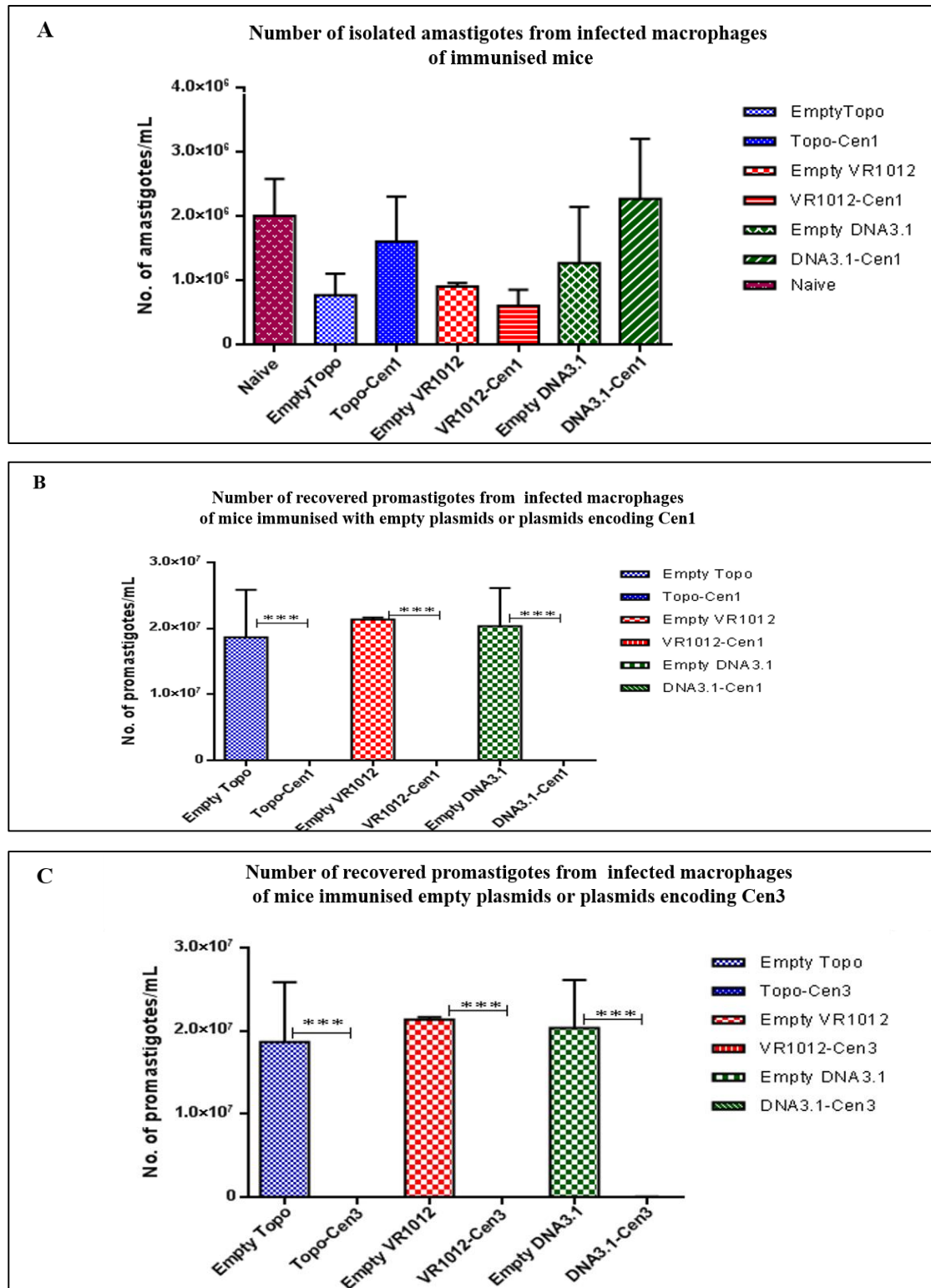


Figure 3.3-6 The expression of macrophage markers on the surface of bone marrow-derived macrophages (BM-DMs)

Bone marrow cells were flushed out of the hind limbs of immunised and control mice using serum-free medium. Cells were cultured in the presence of 1µg per ml GM-CSF at 37°C in a CO₂ incubator. The cells were washed with fresh DC medium every two days. On day 6 adhered cells were further stimulated by incubation with 1µg LPS overnight. Cells were immunostained with cell surface markers and analysed by flow cytometry.

3) *Leishmania* DNA constructs immunogenicity



3) *Leishmania* DNA constructs immunogenicity

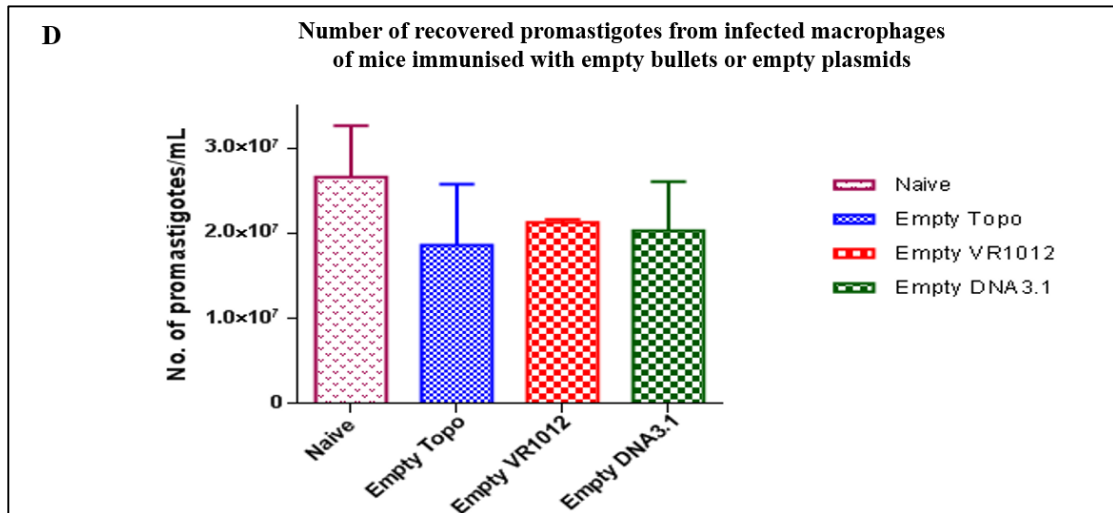


Figure 3.3-7 Survival assay

Balb/c BM-macrophages were infected with *L. mexicana* promastigotes for 48 hours. Amastigotes were isolated by homogenising the infected cells. Graph (A) shows there was no difference in the number of amastigotes which were isolated from infected macrophages of mice immunised with empty bullets, empty plasmids, or plasmids encoding *L. donovani* centrin1, (similar results were obtained from macrophages generated from mice immunised with plasmids encoding *L. donovani* centrin3). Graphs B and C show the number of recovered promastigotes obtained from cultures of amastigotes isolated from macrophages of mice immunised with empty plasmids versus plasmids encoding *L. donovani* centrin1 or *L. donovani* centrin3, respectively, in Schneider's medium supplemented with 20% v/v HIFCS over a period of two weeks. Graph (D) show there was no difference in recovered promastigotes obtained from cultures of amastigotes isolated from macrophages of naïve (empty bullets) or empty plasmids immunised mice. The results represent the average of three experiments for each DNA construct. ***P <0.001 using the Student's t-test. The comparison was performed between plasmids encoding *Leishmania* genes and empty plasmids. Data are presented as mean + SEM.

3) *Leishmania* DNA constructs immunogenicity

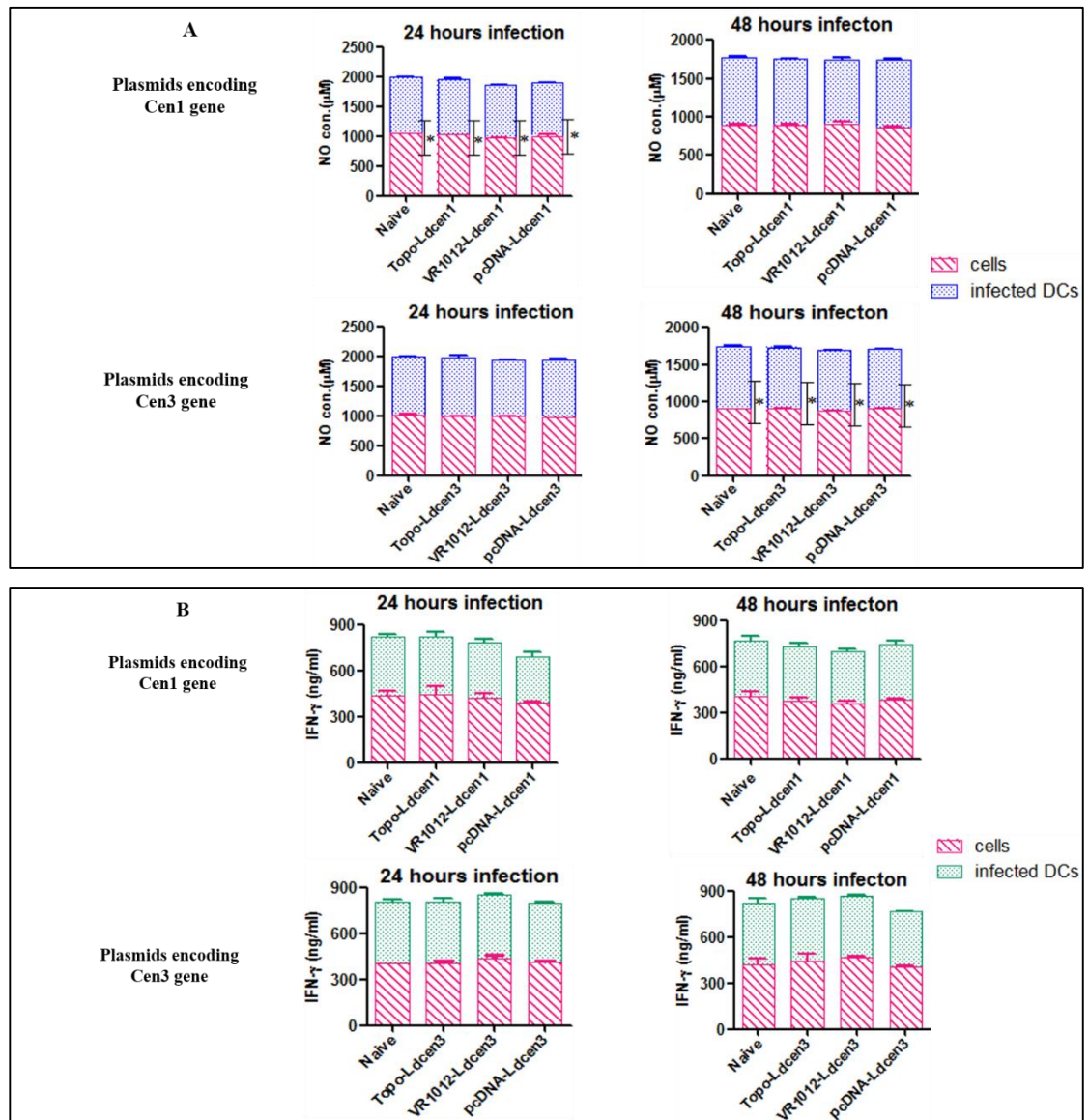


Figure 3.3-8 NO and IFN- γ analysis in infected macrophages

Bone marrow macrophages generated from immunised mice were infected with *L. mexicana* promastigotes. Supernatant from infected and non-infected cells was collected at 24 and 48 hours. Collected samples were analysed for nitrite and IFN- γ concentrations (**A**) and (**B**), respectively. The graphs are the average of three experiments for each DNA construct. * $P < 0.05$ using the Student's t-test. Data are presented as mean + SEM.

3.4 Discussion

One of the objectives of this study was to construct three *Leishmania* genes in three different plasmids, and use them as a delivery vehicle to compare their immunogenicity. Each of the *L. mexicana* gp63, *L. donovani* centrin1 and *L. donovani* centrin3 genes were sub-cloned into pcRT7/CT-TOPO, VR1012, and pcDNA3.1/Hygro(-) plasmids. Both VR1012 and pcDNA3.1/Hygro(-) contain kanamycin and ampicillin-resistance genes, respectively, and they are eukaryotic expression plasmids, due to the presence of cytomegalovirus CMV promoter (Garmory, *et. al.*, 2003). Although the pcRT7/CT-TOPO plasmid was known as a bacterial expression vector, it has a T7 promoter. However, the ability of this plasmid to express encoded genes in mammalian cells has been demonstrated by Asteal (2011). Sub-cloning of *Leishmania* genes into the chosen DNA plasmids was performed by a series of steps which started by growing *E. coli* competent cells, and bulking of original vectors containing target genes. In direct cloning, the target gene was cut out of the original plasmid using suitable restriction enzymes producing sticky ends to increase the ligation efficiency. In the indirect cloning, the target gene was amplified by PCR using a specific primer with the required restriction enzyme sequence. The recognition sites of the chosen restriction enzymes should not present in the sequence of any of the target genes. To achieve high ligation efficiency, the empty plasmids were dephospholigated to prevent self-ligation. The samples (genes and plasmids) were mixed with T4 DNA ligase enzyme and incubated overnight to ensure proper incorporation of the insert (target gene) into the target plasmid. All selected *Leishmania* genes were successfully cloned into the chosen plasmids. It has also been shown that no base miss-matching was detected during the gene cloning as determined by sequencing. The transfection results of the MCF-7 cell line illustrated the ability of *Leishmania* DNA constructs to induce gene expression and protein synthesis in transfected cells compared to non-transfected cells (Figure 3.1.7), and these results confirmed the previous finding reported by Rezvan (2007) and Asteal (2011). Further experiments illustrated that there is no significant difference between the level of expression of the target genes between human MCF-7 cell line and murine CT-26 cell line (data not shown).

Among the different immunisation methods, gene gun immunisation has been widely used (Sakai, *et. al.*, 2000, Dumonteil, *et. al.*, 2003). Two immunisation methods, gene

3) *Leishmania* DNA constructs immunogenicity

gun, and intramuscular inoculation immunisation, were compared (Rezvan, 2007). The results showed that immunisation of Balb/c mice with 100µg of pcDNA3.1-L.mexgp63 DNA construct by intramuscular inoculation and the same construct coated on gold particles and delivered using gene gun method, a good immune response was induced using the both methods. Although the DNA concentration used in the gene gun method was considerably less compared to the amount used for intramuscular inoculation, 66% and 33% of immunised mice were lesion-free for gene gun and intramuscular immunisation, respectively. Therefore, the gene gun immunisation method was chosen in the current study to immunise susceptible Balb/c mice.

Different vaccines can induce different immune responses, which vary according to the method of administration and immunisation schedule (Liu, *et. al.*, 2005), and different *Leishmania* species required different immune responses. It is believed that the Th1 immune response is the key factor in the control of *Leishmania* spp infection, therefore, this type of immune response is the ultimate requirement of a good vaccine to Leishmaniasis (Sacks and Noben-Trauth, 2002). IFN-γ secretion was evaluated in this using Elispot assay, and the results clearly showed significant differences in the number of IFN-γ spots secreted by splenocytes harvested from immunised mice with *Leishmania* DNA constructs compared to splenocytes harvested from controls. However, the results illustrated that there were no difference using different plasmids encoding the same gene. In addition, the levels of secreted IL-2, IL-4, and IFN-γ were also evaluated in the supernatant of splenocytes stimulated with *L. mexicana* SLA using ELISA method, and obtained results showed no differences between controls and immunised mice (Appendix 7).

The selection of crude SLA preparation for stimulation in proliferation and Elispot experiments, but not peptides was based on the findings of our previous work where four potentially immunogenic GP63 peptides were predicted using SYFPEITHI software (TPH, A3, A4, A5, A6) and tested by immunisation of Balb/c mice and *in vitro* stimulation, and none of these four peptides were immunogenic (Rezvan, 2007). To search for a suitable immunogenic peptide for each of the three genes using similar approaches will take a considerable time if it is at all successful.

To evaluate Th1/Th2 immune responses after immunisation using the gene gun method, specific antibody responses were estimated by ELISA method to evaluate Th1 and Th2 responses in immunised Balb/cmice. The results of this study showed that there were

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no differences in the total IgG response, however, there was a significant difference in the levels of IgG1 and IgG2a isotopes in response to immunisation with DNA constructs has been reported that the Th1 may reflect the inability of this assay to detect small changes in the total IgG levels versus its isotopes. It is known that the Th1 immune response is characterised by large increases in the levels of IgG2a, IgG2b and IgG3 subclasses, whereas the Th2 immune response is associated with the production of IgG1 and IgE (Su and Stevenson, 2002). Results from this study show that both *Leishmania* specific IgG2a and IgG1 antibody isotypes were detected in the serum of mice immunised with the different plasmids encoding different *Leishmania* genes. This has revealed that *Leishmania* DNA constructs and the method used in this study have succeeded in activating the immune response. The rise of the IgG response is usually associated with protection against infections, but *Leishmania* parasites can also use it to evade the immune response. Coating of *Leishmania* amastigotes by IgG antibodies and binding to macrophages through FCR II enhances the production of the cytokines associated with Th2 immune responses such as IL-10 (Kane, *et. al.*, 2001; Buxbaum, *et. al.*, 2005). The role of IL-10 in disease progression has been demonstrated in the IL-10 knockout mice model, which become resistant to *L. mexicana* infection, and these mice showed a high level of IFN- γ , compared to wild type mice, which developed chronic lesions (Buxbaum, *et. al.*, 2005). Chu, *et. al.*, (2010) reported that IgG1 and IgG2a/c can enhance the production of IL-10 from infected macrophages *in vitro*, through the macrophages Fc γ RIII and Fc γ RI receptors, respectively, and their results also demonstrated that mice lacking IgG1 were more resistant to *L. mexicana* infection. This confirmed the fact that the Fc γ RIII receptor is associated with progression of *Leishmania* infection. In contrast, Fc γ RI is associated with infection control.

Although the result of this study showed the rise of Th1 immune response, as measured by the increase in IgG2a antibody isotype response, the results also demonstrated an increase in Th2 immune response which was characterised by increased IgG1 level, this was in agreement with reports by Liu, *et. al.*, (2005).

In spite of the intensive investigations on the role of CD4/CD8 T cells in *Leishmania* infection control, there is still contradiction in the published findings. Flow cytometry results from this study showed both CD4⁺ and CD8⁺ populations were increased after two weeks from the last immunisation in splenocytes compared to the controls. However, in lymph nodes while there was a slight increase in the CD4⁺ population,

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there were no differences in CD8⁺ population profile. For the intracellular pathogens (such as viruses and *Leishmania* spp), antigens will be mostly taken up, processed, and represented through MHC class I and MHC class II, which induce and activate CD8⁺ to kill infected cells and CD4⁺ to produce cytokines. In the case of DNA immunisation, the synthesised antigens will be processed *via* into the MHC class I or MHC class II pathway, as a consequence of which CD8 or CD4 T cells will be stimulated, respectively.

In the current study, the cell-mediated immune response was measured using a proliferation assay in response to relevant *Leishmania* antigens (SLA). Optimisation experiments were first conducted in order to find the optimal cell number and antigen concentration for the best results, which showed that splenocytes harvested from spleens of immunised mice, specifically proliferated as a response to a second stimulation with *L. mexicana* SLA, compared to controls. Interestingly, the results of this study, illustrate that the proliferation rate of cells harvested from the spleens was higher compared to cells harvested from lymph nodes of immunised mice. This observation highlights the importance of APCs and their role in processing of antigens and representing them on their surface, which eventually stimulates splenocyte proliferation. This may be related to the fact that cells population taken from the lymph nodes contain less macrophages compared to cells population from spleens, that have an important role in antigen processing, presentation (Phillips, *et. al.*, 2010).

In this study, a novel macrophage-resistance assay has been developed to assess immunity against *L. mexicana* infection. To the best of our knowledge, this “survival assay” has been used for the first time to investigate the ability of macrophages from immunised mice to resist *L. mexicana* amastigote transformation. The results of this assay clearly illustrate the potential of *Leishmania* DNA constructs, since amastigotes isolated from immune macrophages were not able to transform to the promastigote stage compared to non-immune macrophages (isolated from control mice or mice immunised with empty plasmids). Although this has been performed on macrophage progenitor cells, which demonstrated resistance to infection *in vitro*, this needs to be confirmed using mature macrophages from immunised animals by peritoneal lavage. In addition, more investigations are needed to determine the effect of *Leishmania* DNA constructs on the amastigote stage in infected macrophages, since the results of this study showed that there is no difference in IFN- γ and NO secretions of infected

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macrophages in immunised mice compared to controls. It is also worth investigating other *Leishmania* species such as *L. donovani*, which target the bone marrow macrophages as a final host.

Collectively, this investigation has addressed the complexity of the immune response to *Leishmania* parasite infection, which seems not only species dependent, and or the genetic background of the host cells, but it also dependent on the vaccine type and immunisation schedule.

Chapter 4/ *L. mexicana* promastigote growth characterisation

4. *L. mexicana* promastigote growth characterisation

4.1 Introduction

Understanding the biological complexity of *Leishmania* parasite is essential for its control (Wheeler, *et. al.*, 2011). The life cycle of *Leishmania* spp is complex, and consists of two different stages promastigote and amastigote, that survive and multiply inside the sand fly and mammalian host, respectively.

Metacyclogenesis is the process whereby *Leishmania* parasites transform from infective procyclic promastigotes into infective metacyclic promastigotes, this process naturally takes place in sand fly vectors. It has been reported that metacyclogenesis is characterized by an increase in the parasite ability to infect and survive in mammalian hosts.

Metacyclogenesis has also been shown to occur in cultures of promastigotes *in vitro*, and is associated with morphological changes in size, shape, length and changes in the surface molecules such as LPG and GP63 (Muskus and Marín, 2002).

Early studies have reported that metacyclogenesis of avirulent *L. major* was unusually delayed, compared to promastigotes which recovered from late-stationary-phase cultures, which were able to infect Balb/c mice (Silva and Sacks, 1987). The loss of virulence which is associated with frequent subculture could also induce metacyclogenesis delay. A study by the same group reported that over 90% PNA positive (the lectin peanut agglutinin a method used as metacyclogenesis indication)-promastigotes was obtained during passage 1, which was down regulated to 10% PNA-promastigotes at passage 94.

Growth conditions can also affect parasite metacyclogenesis. A study by Bates and Tetley, 1993, reported that culturing *L. mexicana* at pH 5.5 in Schneider's *Drosophila* medium supplemented with 20% v/v FCS produced homogeneous stationary phase, and they were resistant to complement-mediated lysis and highly infective to peritoneal macrophages *in vitro*. However, growing the *L. mexicana* promastigotes at pH 7.0 in Schneider's *Drosophila* medium supplemented with 20% v/v FCS produced morphologically mixed populations of stationary phase and were less infectious when they tested *in vitro*.

4) *L. mexicana* promastigote growth characterisation

It is well established that many parasites such as *Plasmodium* and *Leishmania* species have evolved a number of mechanisms that allow them to misdirect the human immune system (Dey, *et. al.*, 2013). Moreover, the size and genetic complexity of the different stages of these parasites allows them to induce a variety of antigens (proteins) that can be detected by the human immune system, and some parasites can also develop into different stages even inside the final host.

The *L. mexicana* life cycle is a complex process (Figure 1.2.2). A part of it occurs inside the midgut of an infected sand fly, where procyclic promastigotes replicate and transform into human-infective metacyclic promastigotes, which are found in the upper gut of the sand fly vector. A second part occurs inside the mammalian host (Bates, 2007). In the current study, it was possible to reproduce the growth of several *Leishmania* spp outside the sand fly using different types of growth media and supplements under laboratory conditions. Reports show that the procyclic forms inside the sand fly correspond to promastigotes in the *in vitro* exponential phase of growth, and that the metacyclic form represents the stationary phase of the *in vitro* culture, which is described as the infectious stage for experimental applications (Sacks and Perkins, 1984; Sacks and Perkins, 1985).

Despite many attempts, including the use of attenuated parasites, or subunits vaccines, (Daneshvar, *et. al.*, 2003), research has failed to develop an effective *Leishmania* vaccine to date. The immunogenicity of several attenuated *Leishmania* spp, has been reported, such as *L. tropica* (Handman, *et. al.*, 1983), and *L. major* (Kimsey, *et. al.*, 1993). Attenuated parasites can be prepared using different methods, such as the knockout of some virulence genes. For example, Alexander, *et. al* (1998) produced attenuated *L. mexicana* promastigotes by knockout of the cysteine protease genes. Another method is to induce attenuation by exposing the parasite culture to antibiotics such as Gentamicin at a known concentration for period of time (Moreira, *et. al.*, 2012). The mechanism of how this antibiotic affects parasite virulence is not known. However, in bacteria, it has been reported that the Gentamicin affects growth by interfering with protein synthesis through the misreading of mRNA, which results in synthesis of unwanted or abnormal protein that ultimately caused the bacterial death (Daneshvar, *et. al.*, 2003). Whether or not this occurs in *Leishmania* is uncertain.

In the current study, the effect of laboratory conditions on the growth of *L. mexicana* promastigotes was investigated, and their attenuation was carried out naturally by serial

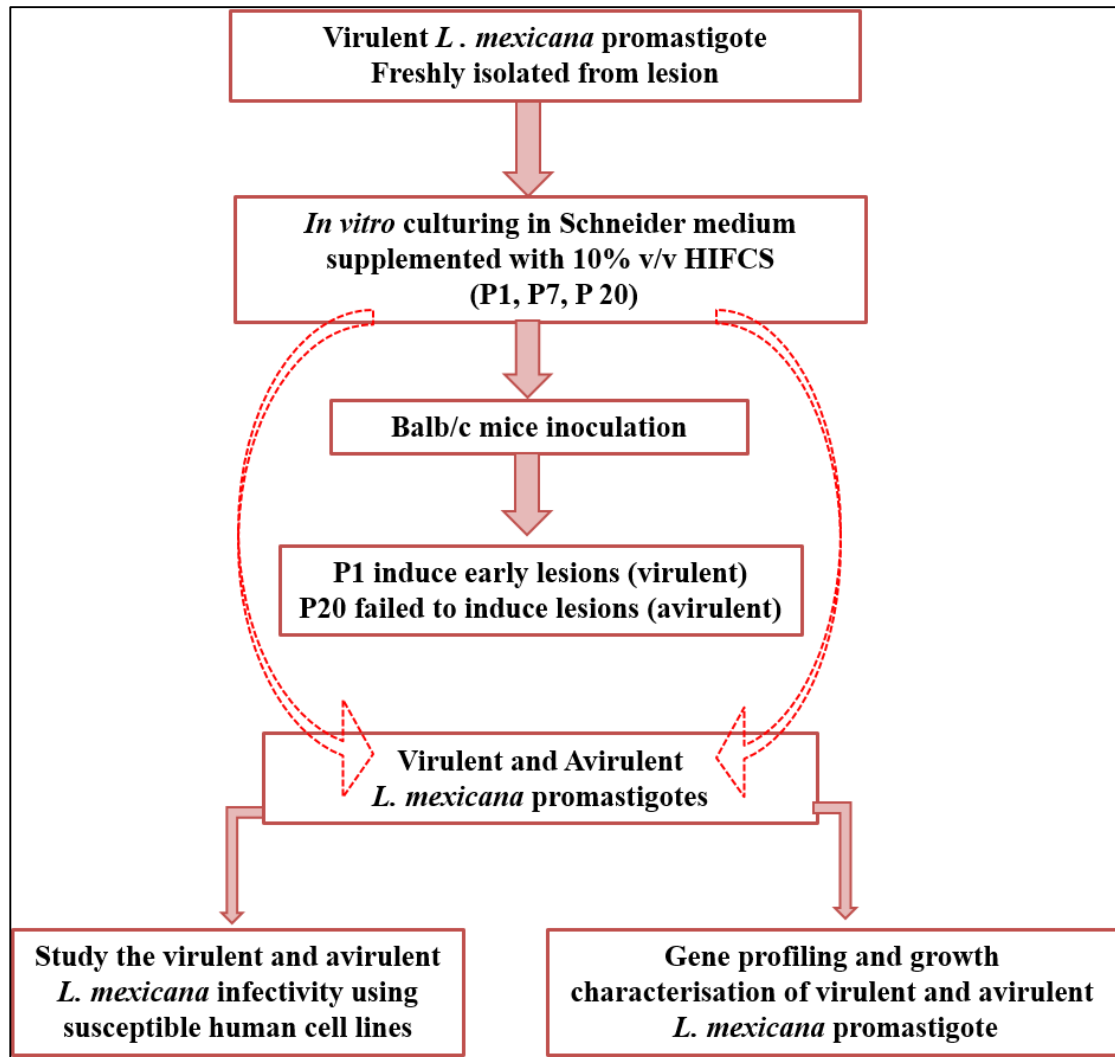
4) *L. mexicana* promastigote growth characterisation

in vitro passaging of virulent *L. mexicana* (P1) in growth medium without antibiotics. Although phenomenon of losing virulence because of *in vitro* passaging has been previously reported (Mitchell, *et. al.*, 1984). In this study changes in the virulence associated gene expression profile (Oliver *ea. al.*, 2012), parasite infectivity and ability to transform into amastigotes in the host cells have been thoroughly investigated.

4) *L. mexicana* promastigote growth characterisation

The work flow

The experiments of this chapter were carried out according to the following schedule:



4.2 Results

4.2.1 *Leishmania mexicana* identification

A standard ITS1 PCR restriction fragment length polymorphism (RFLP) technique was used to confirm species identity of *L. mexicana* used in this study. In this method the internal transcribed spacer 1 (ITS1) gene encoding for 18S rRNA and 5.8S rRNA was first amplified by PCR, then digested with HaeIII restriction enzyme. Results in Figure 4.2.1 show that bands from the digested PCR product were identical to that published by Schonian, *et. al.*, (2003).

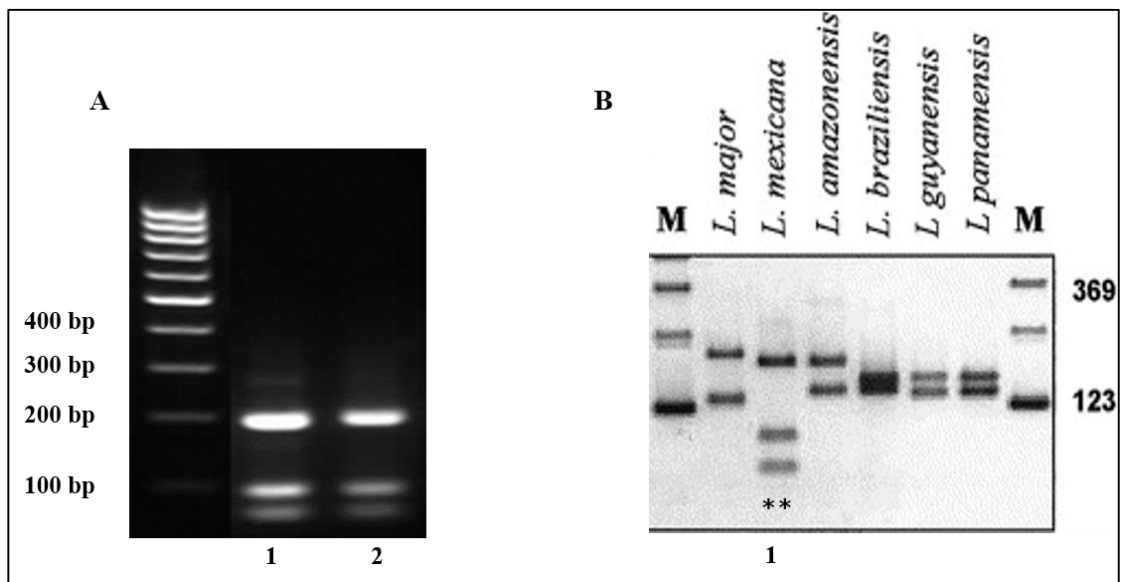


Figure 4.2-1 RFLP analysis to identify *L. mexicana* species

Parasite DNA was extracted from P1 and used as a template to amplify ITS1 (ribosomal internal transcribed spacer 1) region, the PCR product (ITS1) digested with Hae III restriction enzyme. Digested products were separated in 2% w/v agarose gel for 2 hours. The experiment was repeated twice as shown in picture (A). The results were comparable with those published by Schonian *et. al.*, (2003). Picture (B) *L. mexicana* is marked **

4) *L. mexicana* promastigote growth characterisation

4.2.2 *L. mexicana* promastigote growth characterisation

As with other parasite species, *L. mexicana* can be grown in the laboratory and used for *in vitro* investigations, however, due to the effect of different conditions on the parasite infectivity, it is important to determine the growth characteristics of the target species. *L. mexicana* promastigotes were obtained from freshly harvested lesions from infected Balb/c mice as described in the methods section. The harvested parasites were passaged *in vitro* for specific periods of time to investigate the effect of growth on their infectivity.

4.2.2.1 The effect of *in vitro* passage on *L. mexicana* infectivity *in vivo*

In order to investigate the effect of *in vitro* passage on *L. mexicana* promastigote virulence, susceptible Balb/c mice at 6 to 8 weeks of age were inoculated I.D with 2×10^6 *L. mexicana* promastigotes of different passage numbers in a shaved area on the back about 2cm from the tail base. Mice were monitored for lesion development. Results demonstrate that passaging the parasite *in vitro* has caused a gradual loss of *in vivo* infectivity. Virulency of *L. mexicana* promastigotes has been reduced at passage 7, and completely lost at passage 20 (Figure, 4.2.2).

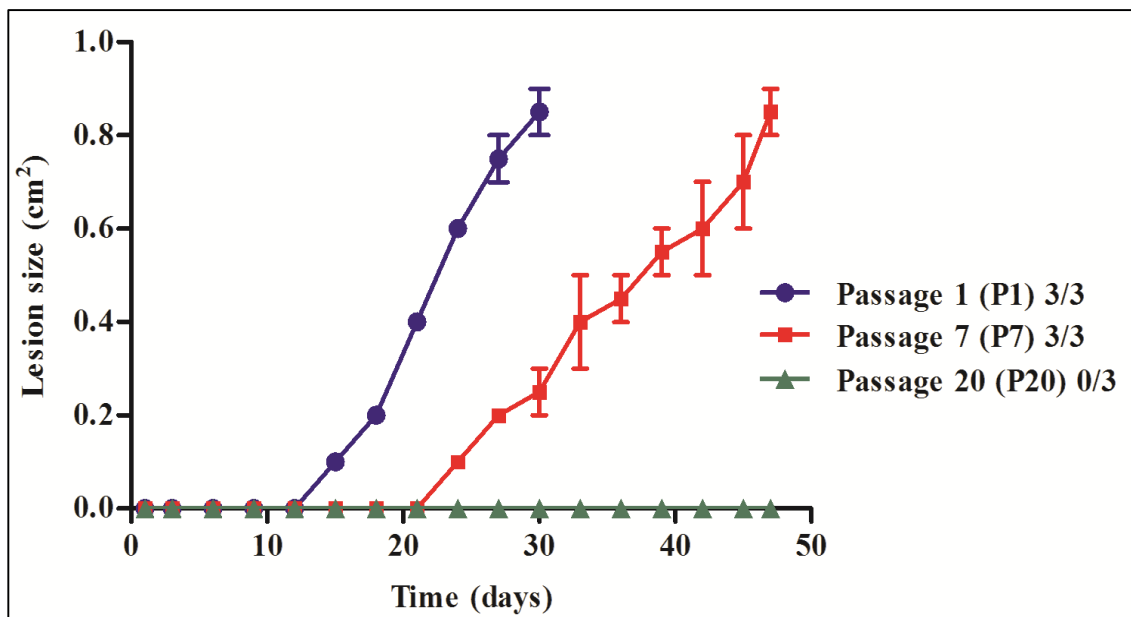


Figure 4.2-2 *In vivo* infectivity of different passage numbers of *L. mexicana* promastigote Groups of three Balb/c mice aged 6-8 weeks were injected I.D with 2×10^6 *L. mexicana* promastigotes at passage 1, 7 and 20. Mice were monitored for lesion development. Data are presented as mean + SEM, and the graph represents three different experiments.

4) *L. mexicana* promastigote growth characterisation

4.2.2.2 The effect of *in vitro* passage on *L. mexicana* population growth

To evaluate the effect of *in vitro* passage on *L. mexicana* promastigote growth, parasites at passages 1, 7 and 20 were cultured anaerobically in Schneider *Drosophila* medium (Lonza, UK), supplemented with 10% v/v HIFCS medium at 25°C, with daily counting using a Neubauer haemocytometer. The exponential growth phase of *L. mexicana* promastigotes at all passages was very short and only lasted for few days. It was also noted that there was no stationary phase of *L. mexicana* promastigotes at any tested passage number. Although there were no significant differences in the growth curve pattern between the different passage numbers, there was a clear difference in the total parasite number at the growth peak, which increased over time from 31 million at P1 to 60 million at P20. In contrast the overall growth curve span, was longer at P1 compared with P20 (Figure 4.2.3).

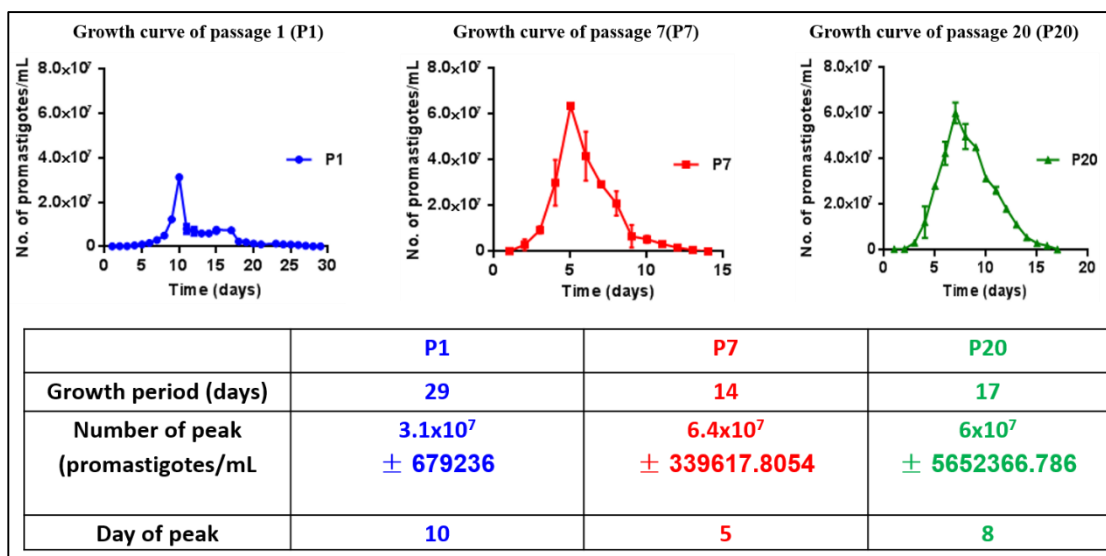


Figure 4.2-3 *In vitro* growth curves of *L. mexicana* promastigotes at P1, P7 and P20

Each growth curve was obtained from daily counting of three sets of cultures which initiated at a concentration of 1×10^6 organisms in 10 ml of Schneider *Drosophila* medium (Lonza, UK), supplemented with 10% v/v HIFCS in T25 flasks at 25°C for the experimental course. Graphs represent at least three independent experiments. Data are presented as mean + SEM.

4) *L. mexicana* promastigote growth characterisation

4.2.2.3 The effect of *in vitro* passage on the morphology of *L. mexicana* promastigote

Another interesting phenomenon was reported in this study, where passaging of *L. mexicana* promastigotes *in vitro* has revealed morphological changes (Figure 4.2.4, top panel). Therefore, it was decided to estimate their body size and flagella length. The promastigotes at late log phase of passages 1, 7 and 20 were measured using a calibrated micrometer slide. Results show a significant increase in cell body size, which was positively related to passage number, however there was no significant difference in flagellum length among *L. mexicana* promastigotes from different passage numbers (Figure 4.2.4 lower panel).

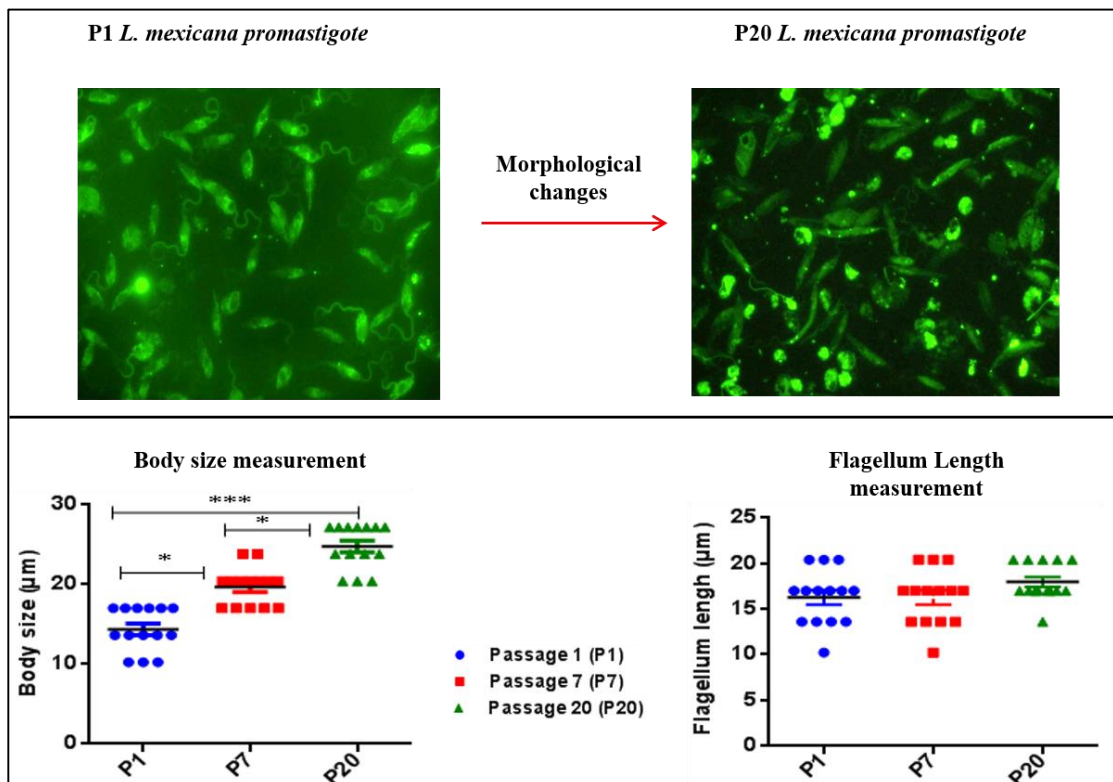


Figure 4.2-4 The effect of prolonged *in vitro* culture on *L. mexicana* morphology

A million promastigotes from P1 and P20 *L. mexicana* promastigotes at late log phase were washed with sterile PBS and stained with 5ng per ml CFSE. Cells were visualised using a fluorescence microscope with a 100x objective (Top panel). The graph shows a significant increase in the cell size, but no difference in flagellum length, as it measured using a calibrated slide, (Lower panel). Graphs represent data from at least three independent experiments, *P<0.05, ***P<0.001 using the Student's t-test. Data are presented as mean + SEM.

4) *L. mexicana* promastigote growth characterisation

4.2.2.4 The effect of *in vitro* passaging on the virulence of *L. mexicana*

To evaluate the effect of *in vitro* culturing on the expression of virulence-associated genes, total mRNA was extracted from the late log growth phase at passages P1, P7 and P20, and converted into cDNA, which was used for qPCR analysis. Results clearly illustrate a significant down regulation of the expression of GP63, LPG2, CPC, CPC2, CPC2.8, Ldcen, and LACK genes (Figure 4.2.5. A). CHT1 gene was also down regulated as result of *in vitro* passage. However, the level of the expression of this gene was restored at passage 20. Further experiments were conducted to determine the effect of *in vitro* passaging on expression of some parasite-related proteins. Immunofluorescence staining using anti-GP63 monoclonal antibodies demonstrated that the expression of GP63 protein on the surface of *L. mexicana* promastigotes was decreased as a result of *in vitro* passaging. The pictures showed the green colour (represent GP63 protein), disappeared over time from P1 to P20 (Figure 4.2.5.B).

4) *L. mexicana* promastigote growth characterisation

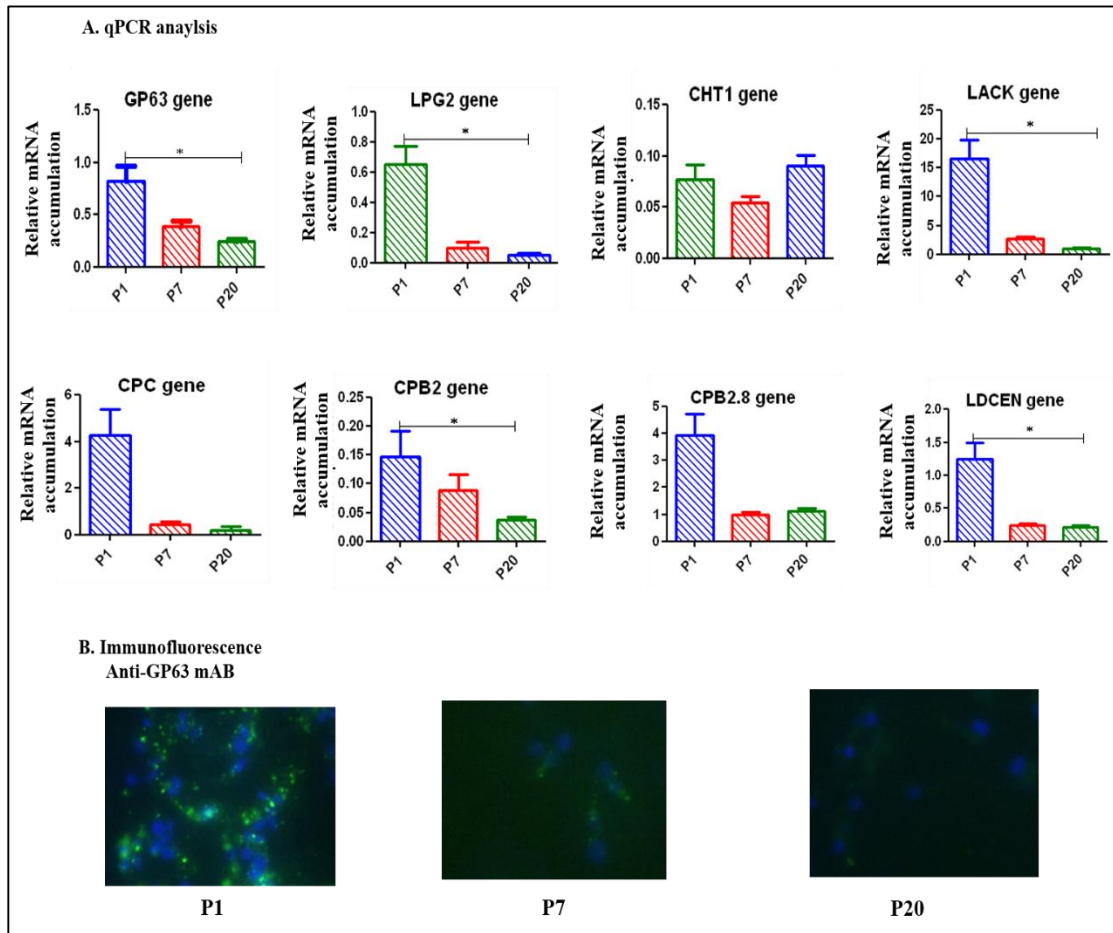


Figure 4.2-5 Gene expression of virulence-associated genes in *L. mexicana* promastigotes at P1, P7 and P20

Total mRNA was extracted from promastigote late log growth phase for each passage number, total RNA was converted to cDNA which was used as a template for qPCR analysis. The qPCR was performed by specific primers for each gene of interest using a Rotor™ 6000 Real Time PCR machine (Corbett Research. Qiagen, Germany). Results were normalized to two housekeeping genes (*L. mexicana*- β tubulin, *L. mexicana*- α tubulin). Results represent three independent experiments. * $P < 0.05$ using the Mann-Whitney test (Panel A). Data are presented as mean + SEM. One million promastigotes of P1, P7, and P20 were stained for GP63 protein localization using anti-GP63 monoclonal antibodies. The pictures illustrate a decrease in the expression of GP63 as a result of *in vitro* culturing, which correlates with the intensity of the green colour (Panel B).

4.2.3 Host cell-*L. mexicana* promastigote interaction

In order to investigate the effect of *in vitro* passaging on *L. mexicana* promastigote infectivity, the infectivity of the promastigotes at passage 1 (virulent), and passage 20 (avirulent) was measured *in vitro* in U937 monocyte and U937 macrophages.

4.2.3.1 Characterization of U937 macrophages

The U937 monocyte target cells were maintained as described in the Materials and Methods (section 2.2.5.1.2). Both PMA and LPS (Sigma, UK) were used separately, at concentrations of 1, 2 and 5 µg per ml as macrophage maturation agents to select the optimum concentration that could be used to induce U937 monocytes into U937 macrophages. Results in Figure 4.2.6. A, clearly show that the use of PMA has produced more adhered U937 macrophages compared with LPS. In addition, the lower concentration, 1 µg per ml PMA, gave a high number of adhered cells in comparison to higher concentrations. Therefore, U937 macrophages obtained from treatment with 1 µg PMA were used for further analysis. Phase contrast imaging using confocal microscopy also shows morphological changes of U937 cells after treatment with 1 µg PMA. The pictures in Figure 4.2.6.B, demonstrate a big cytoplasm and small nucleus in matured macrophages, in contrast to the immature U937 monocyte, which has a big nucleus and small cytoplasm.

4) *L. mexicana* promastigote growth characterisation

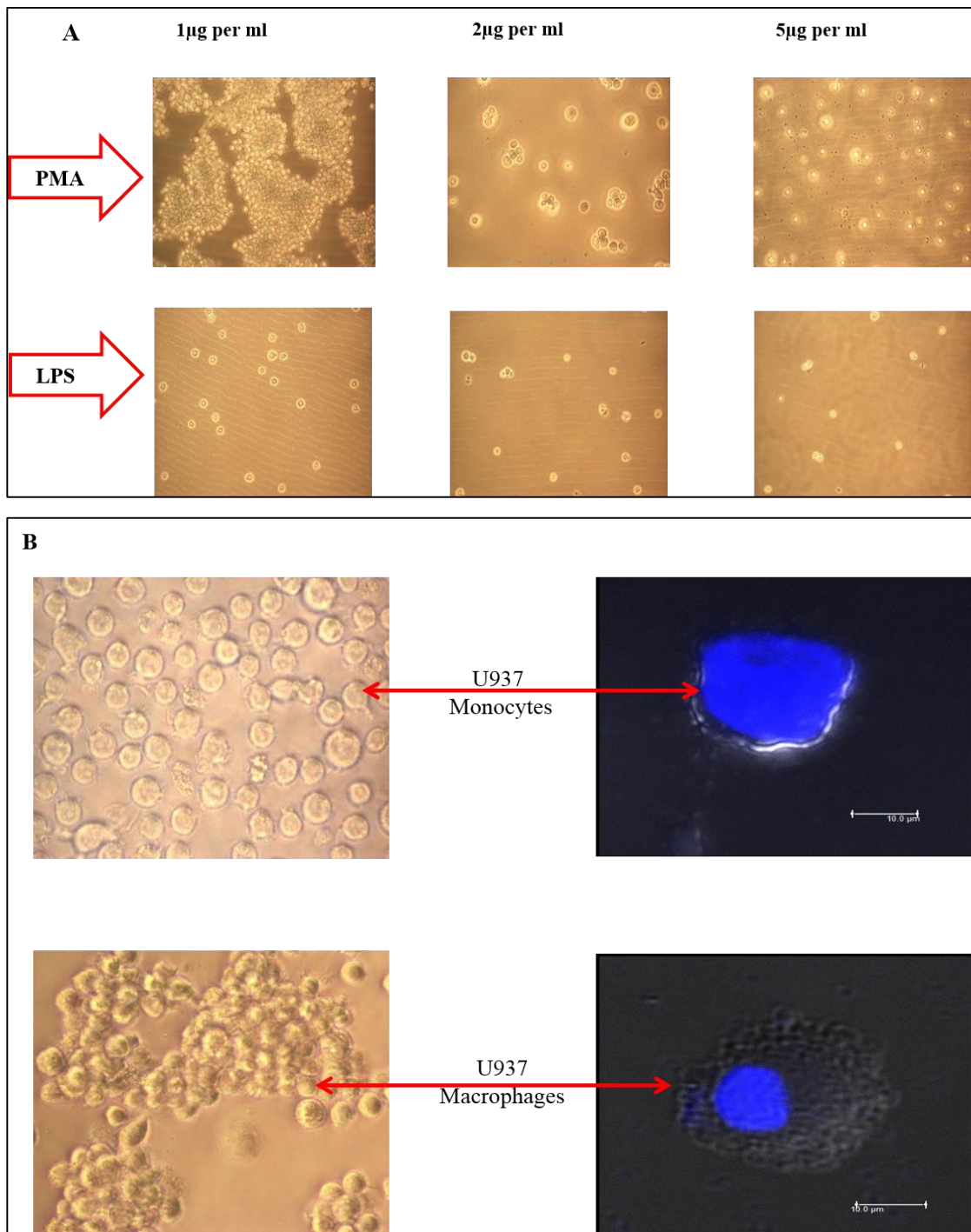


Figure 4.2-6 Characterisation of U937 macrophages

(A) Maturation of U937 monocyte cells with different concentrations of PMA and LPS. U937 monocytes were seeded in 24 well plates at a concentration of 2×10^5 per ml, stimulated with the required concentration of PMA or LPS, and incubated at 37°C for 48 hours. The culture medium was removed and the centres of the wells were visualized and captured using a Nikon Eclipse TS100 microscope connected to a Nikon digital net camera DN100 at 10x magnification. (B) Morphological changes were observed using a confocal microscope, target cells were stained with DAPI. The pictures represent different images from two independent experiments.

4) *L. mexicana* promastigote growth characterisation

The obtained U937 macrophages from U937 monocytes were further assessed for the expression of macrophage markers (CD14, CD68 and T4/80) using qPCR, which was further confirmed by flow cytometry analysis. Total mRNA was extracted from matured cells using 1µg per ml PMA and U937 monocytes (used as a control), and converted into cDNA which was used to check the expression of target macrophage markers. Results in Figure 4.2.7.A illustrate that there was a significant expression of the three chosen macrophage markers on the U937 macrophages compared with U937 monocytes. qPCR results were further confirmed by using flow cytometry analysis, which also showed that 36.48%, 54.78% and 80.54% of U937 macrophages expressed CD14, CD68 and F4/80, in comparison to 1.64%, 3.12% and 1.78% of U937 monocytes, respectively (Figure 4.2.7.B).

Macrophage maturation was also confirmed using the latex beads uptake assay to determine the engulfing capability of matured U937 macrophages. The confocal microscopy results clearly show that there was average of three beads per cell when 1µg per ml PMA was used to mature the U937 monocytes into U937 macrophages, compared with only one or no beads per cell when 2µg or 5µg per ml PMA was used (data not shown).

Collectively, the results demonstrate that using the PMA at a concentration of 1µg per ml was the optimal concentration to induce U937 monocyte maturation to U937 macrophages, which induced more U937 macrophages expressed a high level of all the tested macrophage markers compared with U937 monocytes, and induced high engulfing capability.

4) *L. mexicana* promastigote growth characterisation

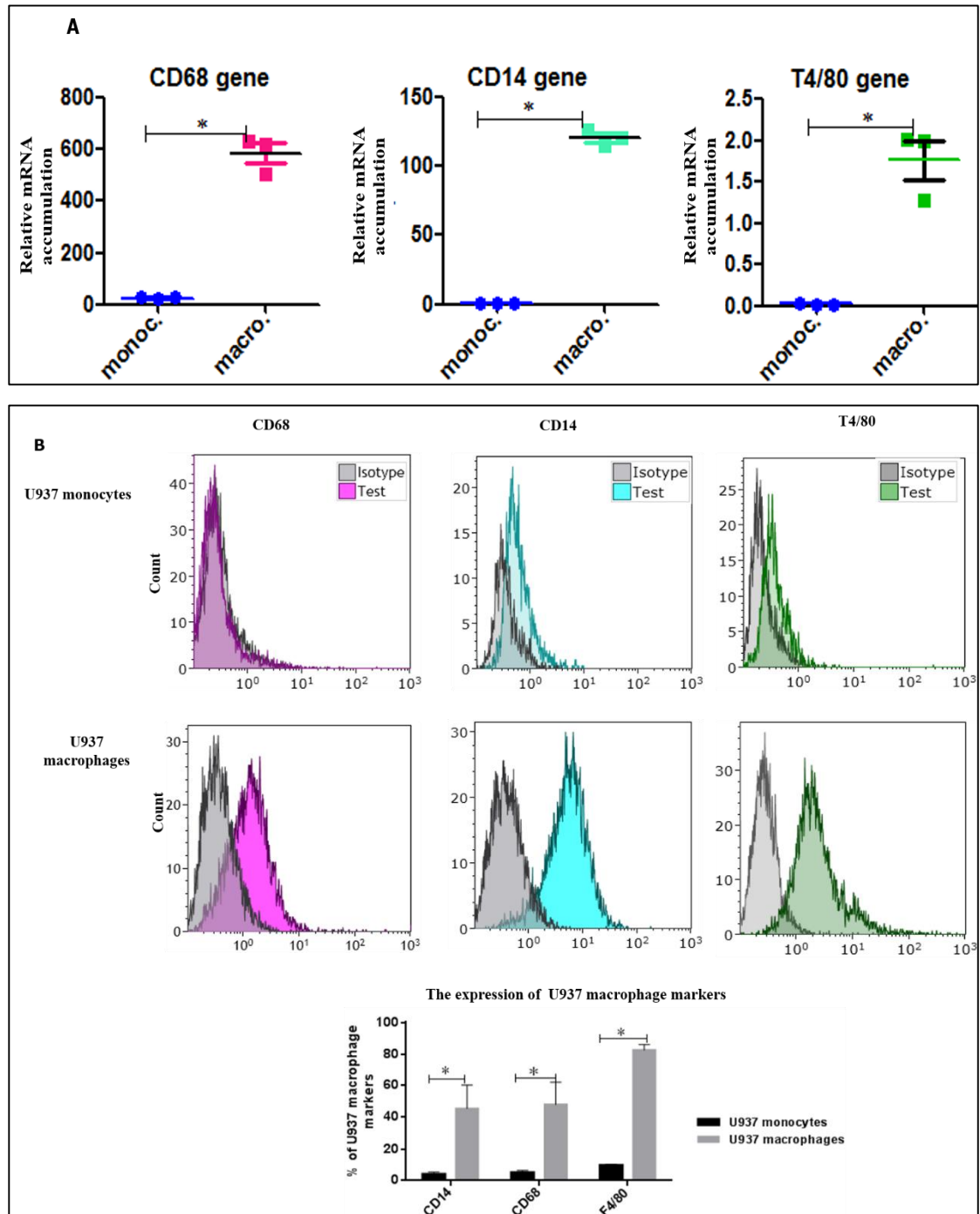


Figure 4.2-7 The expression of U937 macrophage markers

(A) RNA was extracted from U937 monocytes and U937 macrophages, and converted into cDNA for qPCR analysis. Results showed a significant expression of T4/80, CD40 and CD68 markers; the bars represent three independent experiments. (B) Shows histogram of flow cytometry analysis of U937 macrophage markers. U937 macrophages and U937 monocytes were stained for the expression of target markers. Data are derived from at least 3 independent experiments, * $P < 0.05$ was calculated using the Mann-Whitney test. Data are presented as mean + SEM.

4.2.4 Interaction of *L. mexicana* with U937 monocytes and U937 macrophages

In order to study parasite infectivity, host cells (U937 monocytes and U937 macrophages) were seeded in 24 well plates at a concentration of 2×10^5 cells per well. Target cells were infected with the late log growth phase of virulent and avirulent *L. mexicana* promastigotes at a ratio of 1:30 (cell: parasite) and incubated at 37°C in a humid atmosphere in a 5% v/v CO₂ incubator. Cells were monitored at different time points by microscopic examination using a Nikon Eclipse TS100 microscope connected to a Nikon digital net camera (DN100). A number of cellular changes in the two hours' infected culture were observed, reflecting differences between infection of the target cells with virulent (P1) and avirulent (P20) *L. mexicana* promastigotes. The results clearly show the inability of avirulent parasites to infect target cells (Figure 4.2.8, B), where they formed clusters of promastigotes which remained around the target cells. In contrast the virulent *L. mexicana* promastigotes remained as single cells (Figure 4.2.8, A), were evenly distributed around the target cells, and no parasite clusters were observed following virulent parasite infection. Furthermore, the number of free parasites (promastigotes) isolated from U937 macrophages and U937 monocyte cultures infected with avirulent *L. mexicana* promastigote after 2 hours infection was significantly higher compared with those infected with virulent parasites (Figure 4.2.8. C and D), respectively. Free parasites remaining outside target cells were estimated by counting following slow centrifugation. The higher number of free avirulent *L. mexicana* promastigotes remaining outside the host cells may be due to the inability of these parasites to infect the target cells.

4) *L. mexicana* promastigote growth characterisation

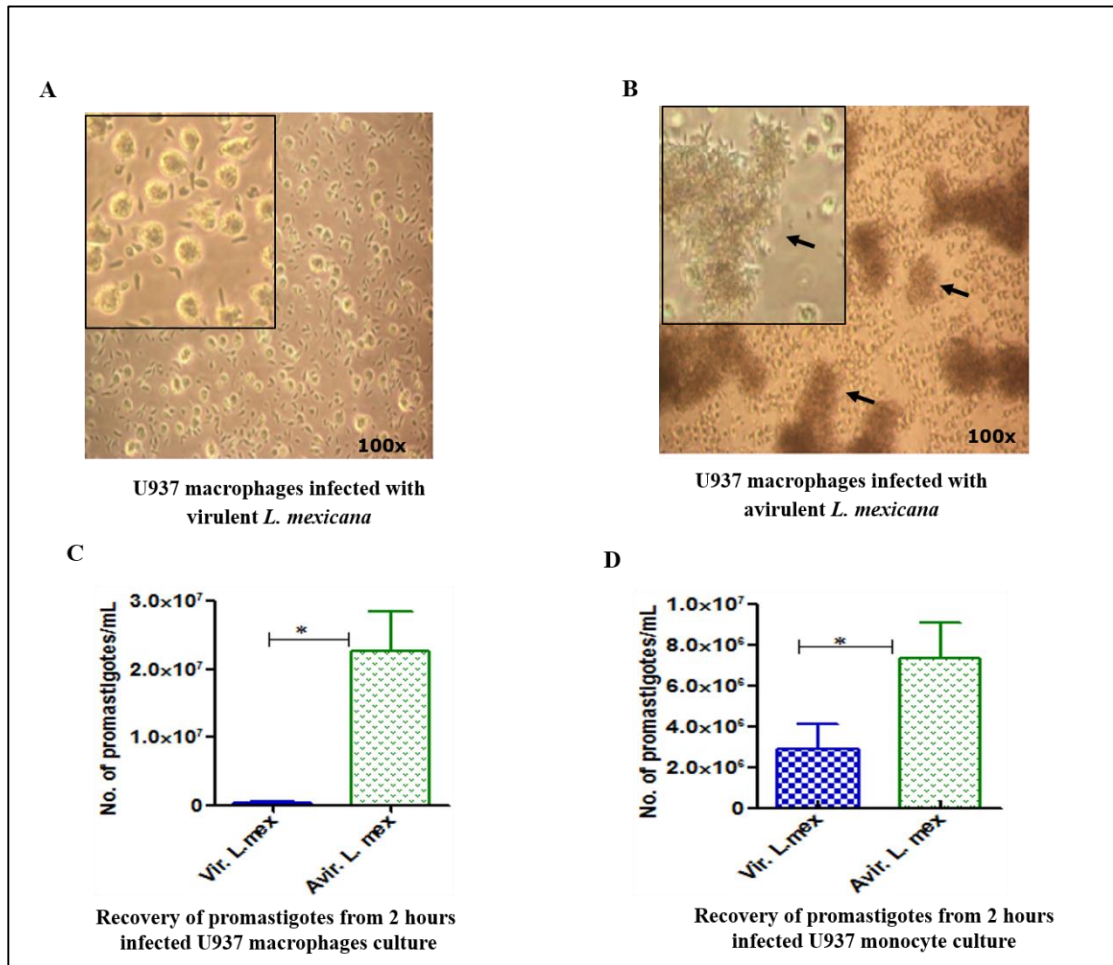


Figure 4.2-8 Interaction of virulent and avirulent *L. mexicana* promastigotes with host cells

U937 macrophages were infected at a ratio of 1:30 (cells: parasite) with virulent (**A**) and avirulent (**B**) *L. mexicana* promastigotes, respectively for 2 hours. Picture (**B**) shows the formation of clusters (indicated by the arrow) produced by avirulent parasites in comparison to the virulent parasite, which remained as single cells adhered to and around the target cells, picture (**A**). The pictures represent more than three experiments using different cell lines, and show the number of free virulent and avirulent *L. mexicana* promastigotes harvested from infected U937 macrophages (**C**) and U937 monocytes (**D**) after infection for 2 hours. Host cells were removed by slow centrifugation, free parasites were counted per ml using a Neubauer hemocytometer. The bars represent 3 independent experiment * $P < 0.5$ using the Student's t-test. Data are presented as mean + SEM.

4.2.4.1 Infection of U937 monocytes and U937 macrophages with *L. mexicana* for 24 hours

The results of infection of target cells with virulent and avirulent *L. mexicana* promastigotes for 24 hours have shown considerable differences in parasite infectivity. Images in (Figure 4.2.9.A) show U937 macrophages infected with virulent and avirulent *L. mexicana* promastigotes for 24 hours. The pictures illustrate that virulent parasites successfully produced amastigotes, which were detected outside the infected cells as the arrow indicates. However, avirulent promastigotes failed to produce significant numbers of amastigotes, and only few of them were detected outside infected cells, the majority of parasites remaining outside the target cells as promastigotes. Amastigote numbers in cultures of U937 macrophages infected with virulent and avirulent *L. mexicana* promastigotes were determined by counting at 2, 5 and 24 hours post infection. Results 4.2.9.A, clearly show a significant number of amastigotes detected after 5 and 24 hours of infection, following the infection with virulent parasites. The amastigote number was highest at 24 hours after infection in comparison to those infected with avirulent *L. mexicana* promastigotes.

A similar trend in results was also observed in the U937 monocyte cell line infected with virulent and avirulent *L. mexicana* parasites (Figure 4.2.9.B). Interestingly, the number of free amastigotes in the U937 monocyte culture infected with both virulent and avirulent parasites was less at 24 hours infection compared with that at 5 hours infection. These results were reproduced using a MonoMac-6 monocyte cell line infected with virulent and avirulent *L. mexicana* promastigotes (data not shown).

4) *L. mexicana* promastigote growth characterisation

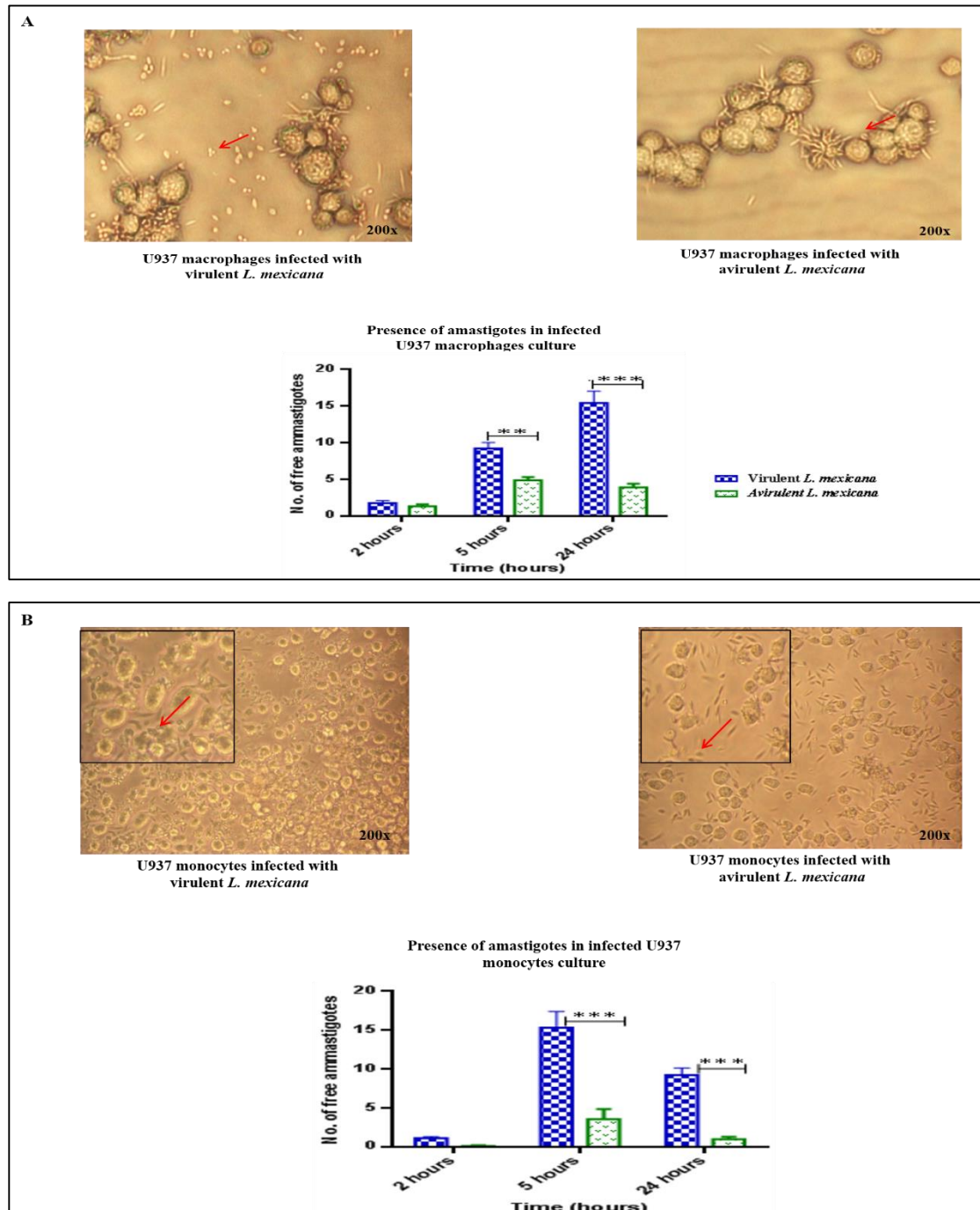


Figure 4.2-9 Infection of U937 macrophages and U937 monocytes with virulent and avirulent *L. mexicana*

Target cells were infected with virulent and avirulent parasites at a ratio of 1: 30 (cell: parasites). Four random microscopic fields from a total of 6 wells per cell line for each experiment were captured using a Nikon Eclipse TS100 microscope connected to a Nikon digital net camera DN100 at different time points (200X magnification). Pictures (A and B) were captured at 24 hours following infection. Graphs (A) and (B) represent the number of amastigotes per microscopic field in infected U937 macrophages, and U937 monocytes, respectively. Bars represent the three independent experiments for each cell line, **P<.01, ***P<.001 using the Student's t-test. Data are presented as mean + SEM.

4) *L. mexicana* promastigote growth characterisation

Similar patterns of results were observed when target cells were infected with virulent and avirulent parasites for 48 hours. Figure 4.2.10.A, illustrates that the avirulent parasites were able to enter the U937 macrophages, but were unable to produce amastigotes, in comparison to the massive production of amastigotes in cells infected with virulent parasites.

To investigate whether virulent and avirulent *L. mexicana* promastigotes can equally infect target cells, a classic method of Giemsa staining and microscopic examination to determine the percentage of infected cells was used. Target U937 macrophages were infected with the virulent and avirulent late log phase of *L. mexicana* promastigotes at a ratio of 1:10 (cell: parasite) for 2, 24 and 48 hours. Infected cells were harvested, smeared onto glass slides and stained with 5% w/v Giemsa stain as described in the Materials and Methods section. The percentage of infected cells when virulent parasites were used steadily increased from 36%, to 50% to 86% at time points 2, 24 and 48 hours, but the percentage of cells infected with avirulent *L. mexicana* promastigotes was 21%, 22% and 38% at 2, 24 and 48 hours (Figure 4.2.10.B). Unlike U937 macrophages, the number of infected U937 monocytes with virulent and avirulent parasites was much less at 48 hours, compared with 24 hours infection (Figure 4.2.10.C). Interestingly, in addition to the differences in promastigote numbers after two hours infection with virulent and avirulent parasites (Figure 4.2.11, top panel), avirulent parasites failed to transform from promastigotes to amastigotes at 24 and 48 hours. The results clearly show that there were few amastigotes per cell in infected U937 macrophages in comparison with the virulent infection (Figure 4.2.11, lower panel).

4) *L. mexicana* promastigote growth characterisation

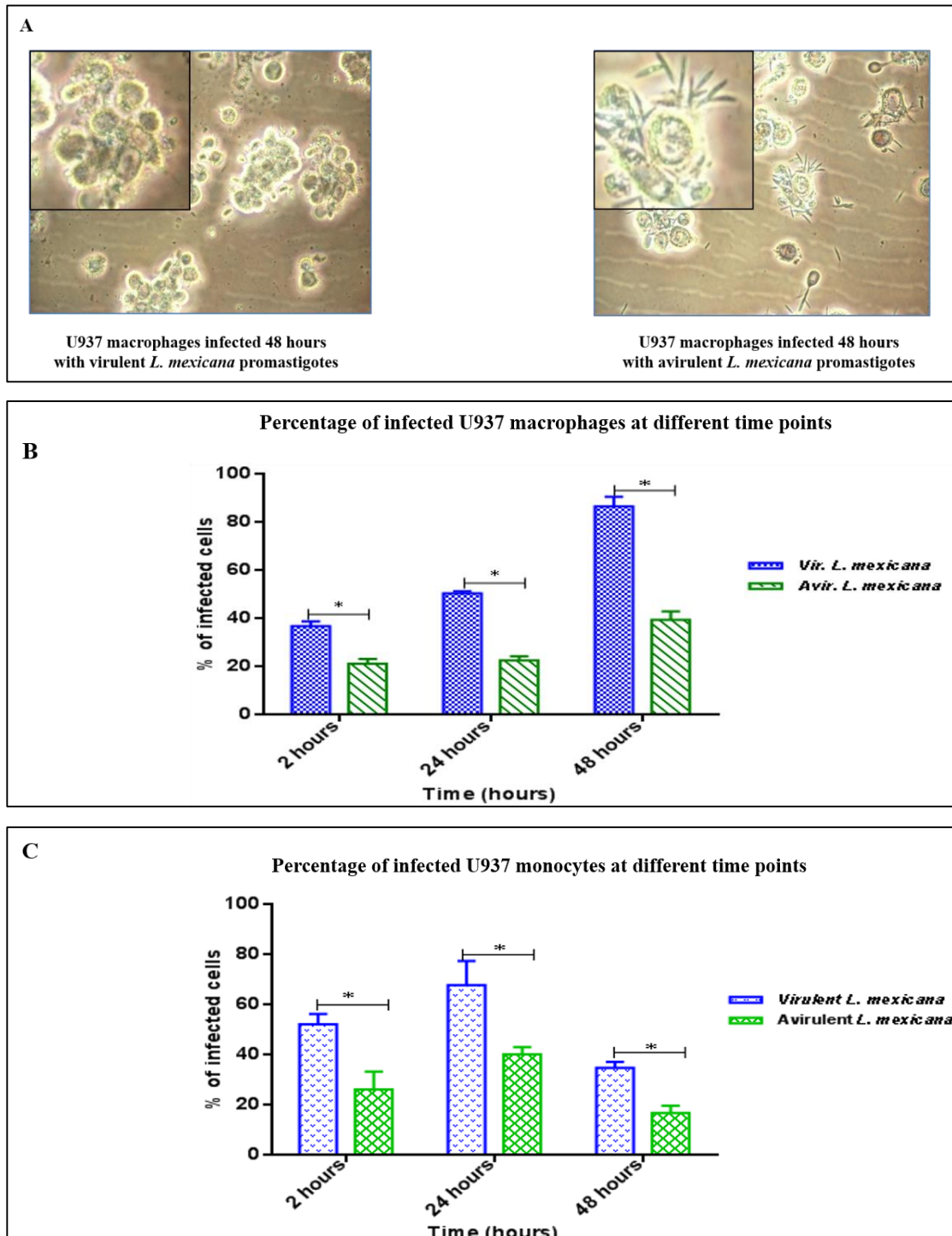


Figure 4.2-10 Interaction of *L. mexicana* promastigotes with U937 monocytes and U937 macrophages 48 hours post infection

Target cells were infected with virulent and avirulent *L. mexicana* promastigotes at a ratio of 1:30 (cell: parasites). Pictures (A) show inability of avirulent *L. mexicana* promastigotes to form amastigotes, in comparison to virulent parasites. The number of infected cells using Giemsa staining shows an increase in the number of infected U937 macrophages over time (B). In contrast, the number of infected U937 monocytes was less at 48 hours infection (C). Data are derived from at least 3 independent experiments, * $P < 0.05$ was calculated using the Mann-Whitney test. Data are presented as mean + SEM.

4) *L. mexicana* promastigote growth characterisation

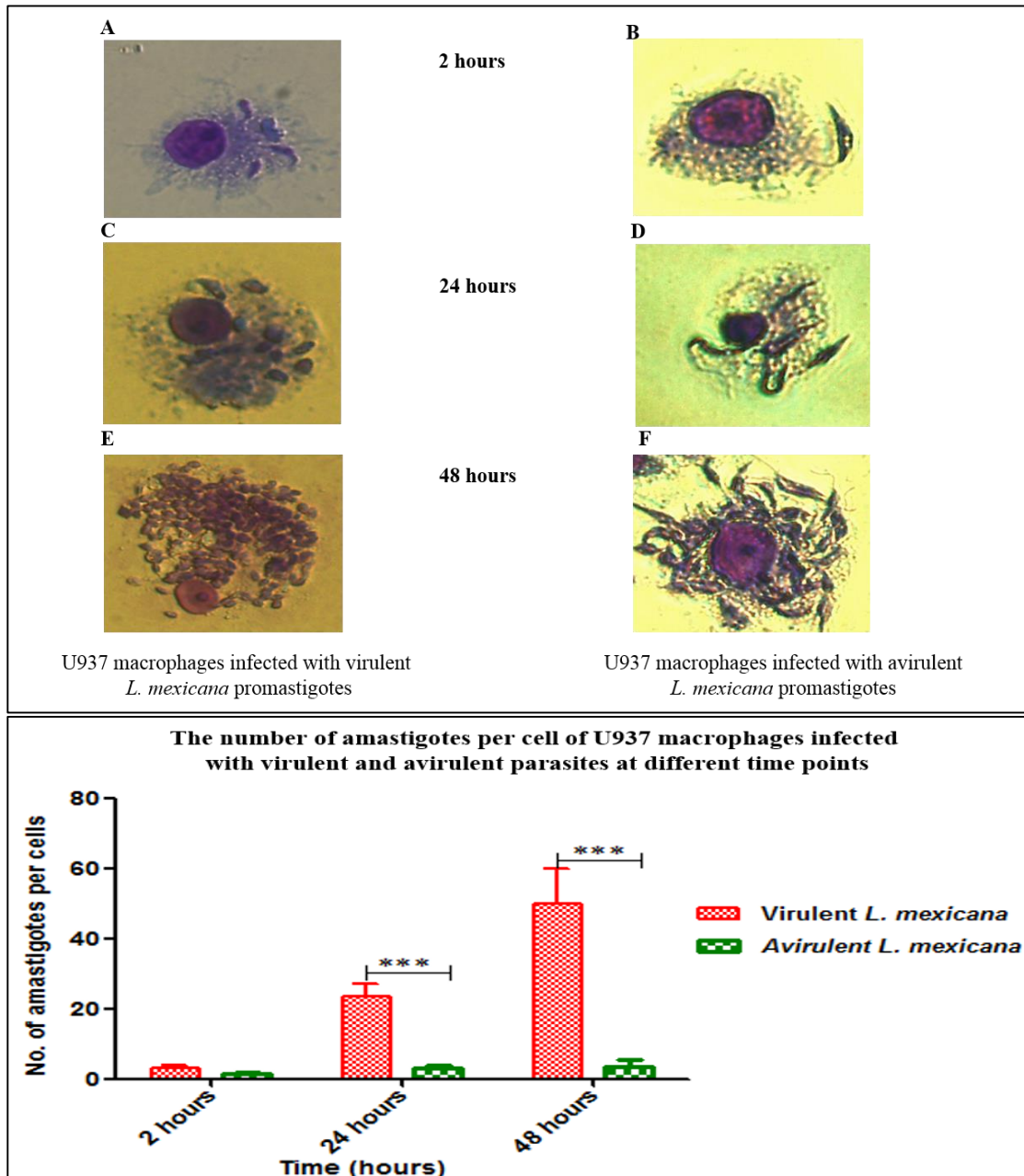


Figure 4.2-11 Interaction of host cells with virulent and avirulent *L. mexicana* promastigotes at different time points

U937 macrophages were infected with virulent and avirulent *L. mexicana* promastigotes 1:10 (cell: parasite), infected cells were stained with 5% w/v Giemsa stain at 2, 24 and 48 hours. The number of virulent *L. mexicana* promastigotes per cell after 2 hours infection was consistently higher in comparison to the number of avirulent promastigotes per cell (A and B, respectively). At 24 and 48 hours, virulent promastigotes were able to transform into amastigotes (C and E, extreme examples of infection). In contrast, avirulent *L. mexicana* promastigotes failed to transform into amastigotes (D and F). *** $P < 0.001$ using the Student's t-test. Bars represent numbers of the amastigotes stage in 100 infected U937 macrophages with virulent and avirulent parasites in two independent experiments. Data are presented as mean + SEM.

4) *L. mexicana* promastigote growth characterisation

4.2.4.2 The effect of conditioned medium on *L. mexicana* promastigote growth

The use of a conditioned medium, derived from U937 monocytes infected for 2 hours with virulent or avirulent parasites, significantly inhibited the growth of avirulent but not virulent *L. mexicana* promastigotes (Figure 4.2.12). The inhibition of avirulent parasite growth was irrespective of whether the conditioned medium was derived from infected U937 monocytes with virulent or avirulent parasite (B and C). A control conditioned medium derived from non-infected U937 monocytes, did not inhibit the growth of either virulent or avirulent *L. mexicana* promastigotes. Although the same pattern of effect on *L. mexicana* growth of conditioned medium derived from infected U937 macrophages, was observed (D, E and F), the results clearly show that the conditioned medium derived from infected U937 macrophages was more supportive to the growth of virulent parasites.

4) *L. mexicana* promastigote growth characterisation

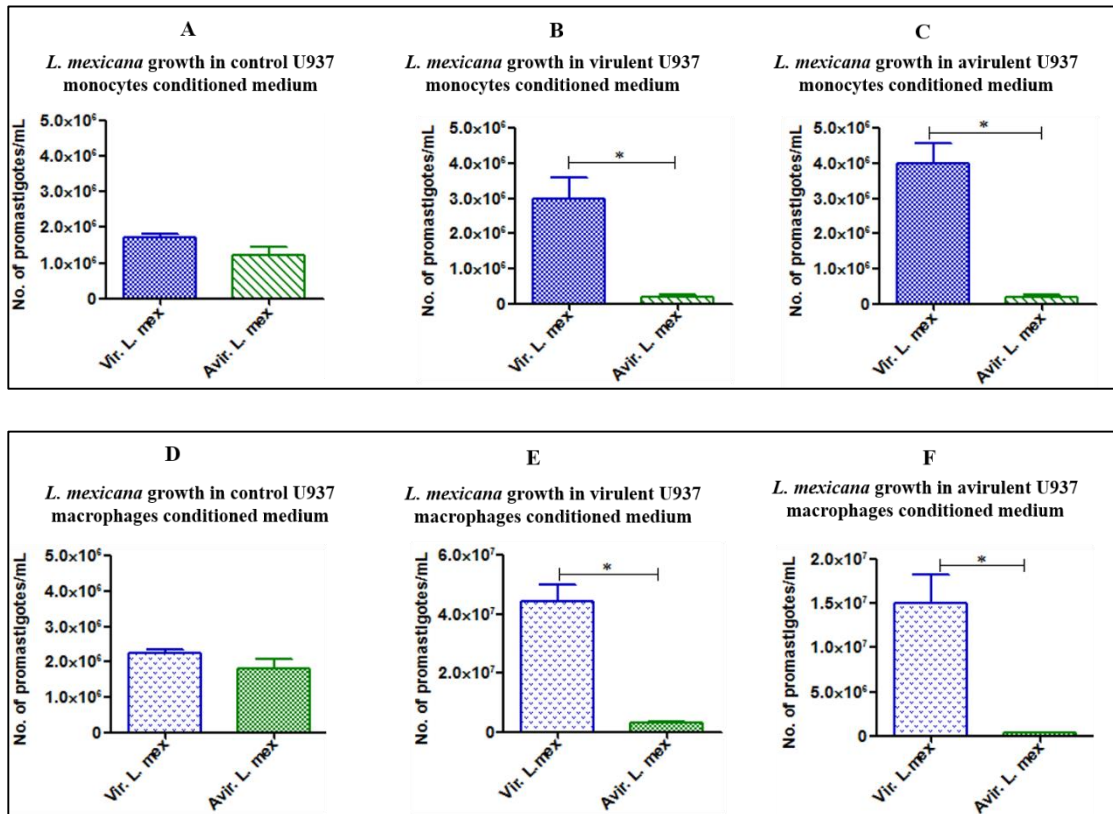


Figure 4.2-12 Effect of conditioned medium on *L. mexicana* promastigote growth
Conditioned medium was produced by infecting target cells for 2 hours. (A) Number of promastigotes after being cultured for 3 days in a conditioned medium of control U937 monocytes. (B) Number of virulent and avirulent promastigotes after being cultured for 3 days in conditioned medium from U937 monocytes infected for 2 hours with virulent *L. mexicana*. (C) Number of virulent and avirulent promastigotes after being cultured for 3 days in conditioned medium from U937 monocytes infected for two hours with avirulent *L. mexicana*. *P<0.05 using the Student's t-test. D, E and F show the effect of the conditioned medium from control and infected U937 macrophages on the growth of virulent and avirulent *L. mexicana* promastigotes. Data are presented as mean + SEM.

4.2.4.3 Host cell lysis induced by parasite infection and A2 expression

The current study has demonstrated that, in spite of the increase in the number of amastigotes after 24 hours of infection in cultures of host cells infected with virulent, but not avirulent parasites, there was no significant difference in numbers of host cells at this time. However, at 48 hours a significant loss in host cell numbers was observed following infection with virulent but not with avirulent parasites (Figure 4.2.13.A). This was further confirmed by the expression of a specific amastigote protein A2 in infected cells, which reflects the parasite load in infected host cells. The qPCR results illustrate that the expression of the A2 gene in U937 macrophages increased from 132 fold at 2 hours, to 360 fold at 24 hours, and dropped to a 201 fold at 48 hours of infection with virulent parasites, compared to 0.09, 0.19, 2.16 fold expression at 2, 24 and 48 hours following the infection with avirulent parasites (Figure 4.2.13.B). The same pattern of A2 gene expression was observed in U937 monocytes infected with virulent parasites (Figure 4.2.13.C). The expression of the A2 gene in U937 monocytes was 73, 152, 32 fold, and 0.015, 0.09, 0.04 fold at 2, 24, and 48 hours when cells were infected with virulent and avirulent *L. mexicana*, respectively.

4) *L. mexicana* promastigote growth characterisation

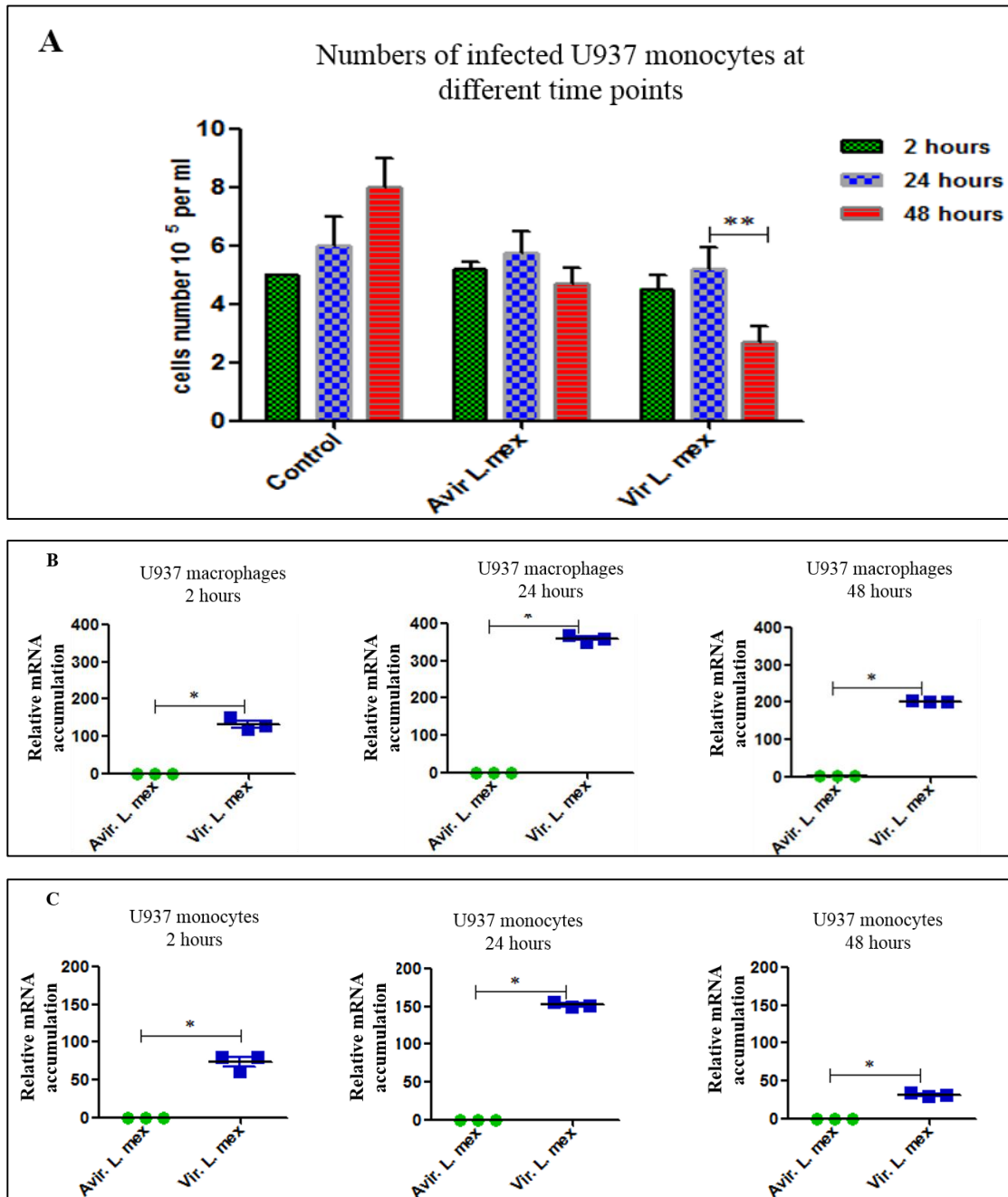


Figure 4.2-13 Infected host cell lysis and amastigote A2 expression

U937 monocytes were infected with virulent and avirulent *L. mexicana* promastigotes at a ratio of 1:10 (cell: promastigotes). Cells were counted using trypan blue at 2, 24, 48 hours post infection. ** $P < 0.01$ using the Student's t-test. The bars in (A) represent three independent experiments. In each experiment six replicates were used. In other experiments, cells were infected for 2, 24, and 48 hours and subjected to mRNA extraction. Synthesised cDNA was used to check the expression of the specific A2 amastigote gene. (B) illustrates A2 expression in infected U937 macrophages and (C) shows A2 gene expression in infected U937 monocytes. Data are derived from 3 independent experiments, * $P < 0.05$ was calculated using the Mann-Whitney test. Data are presented as mean + SEM.

4) *L. mexicana* promastigote growth characterisation

4.2.4.4 Effect *L. mexicana* infection on the phagosome maturation markers

The results of this study show a significant difference between virulent and avirulent *L. mexicana* infection on the expression of Rab7 and Rab9 markers. There is an over expression of Rab7 in U937 macrophages infected with avirulent parasite at all-time points, compared with infection with the virulent parasite (Figure 4.2.14.A). The results also demonstrate that, the expression of Rab9 was up regulated following infection of target cells with the avirulent parasite, but only at 2 and 24 hours of infection. Unexpectedly, Rab9 was up regulated, but not significantly, at 48 hours in host cells infected with the virulent parasite (Figure 4.2.14.B).

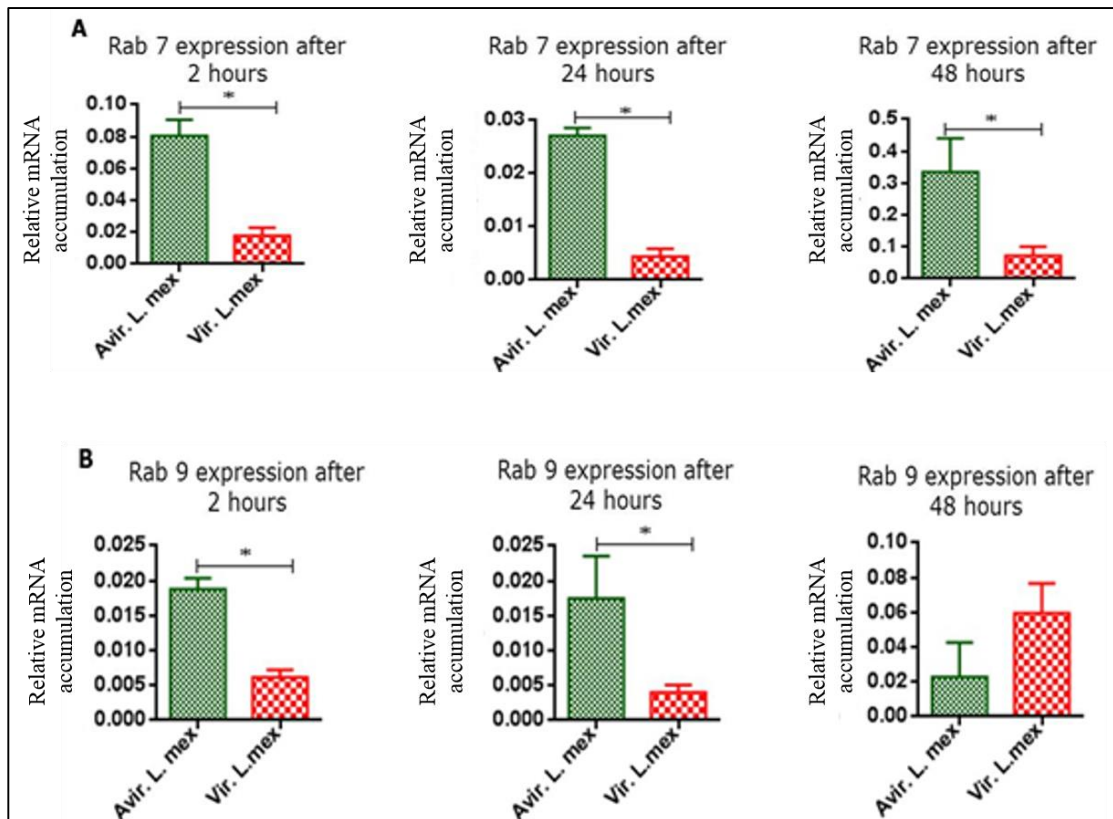


Figure 4.2-14 The expression of phagosome maturation markers in infected U937 macrophages

U937 macrophages were infected at a ratio of 1:10 (cell: parasites), with virulent and avirulent *L. mexicana* promastigotes. Infected cells were then incubated at 37°C and 5% v/v CO₂. Control (non-infected cells) and infected cells were subjected to RNA extraction after 2, 24, and 48 hours; synthesised cDNA was used as a DNA template in qPCR analysis. (A) Represents the expression of Rab7, (B) represents the expression of Rab9, at different time points. Bars represent three independent experiments. *P<0.05 using the Mann-Whitney U test. Data are presented as mean + SEM.

4.3 Discussion

The challenges of *Leishmania* vaccine development are associated with the complexity of the immune response to *Leishmania* spp, which is directly linked to the biological complexity of the two stages of the parasite's life cycle. In this study, the biology of *L. mexicana* promastigotes growth was analysed. Attenuated *L. mexicana* promastigotes were produced naturally by continuous *in vitro* passaging in Schneider *Drosophila* medium (Lonza, UK), supplemented with 10% v/v HIFCS media, without any antibiotics. The infectivity of *L. mexicana* promastigotes at passages 1, 7 and 20 was analysed by intradermal inoculation of Balb/c mice. This study demonstrated that *L. mexicana* parasite infectivity was lost during continuous culture *in vitro*. The loss of virulence was detected at P7, while complete loss was obtained at P20 (Figure 4.2.2). Subsequently, parasites at passage one have been called “virulent”, while the parasites at passage 20 are called “avirulent”. These results were in agreement with the findings of Segovia, *et. al.*, (1992) who reported the loss of virulency in 12 clones isolated from a single *L. major* strain after *in vitro* culturing for two months, and that these clones were unable to form lesions in injected Blab/c mice. The loss of infectivity was also reported in *L. donovani* following long term culture *in vitro*, which was also characterized by the high expression of galactosyl transferase, an enzyme that was absent in infectious *L. donovani* promastigotes (De and Roy, 1999).

The qPCR results of avirulent parasites showed extreme down regulation of almost all tested virulence associated genes. GP63, LPG, and cysteine proteases, which have an important role in parasite-host cell interaction, attachment, internalisation, and survival within the host cells (reviewed in the introduction section 1.3). To the best of our knowledge, this study is the first to report a correlation between promastigote size and virulency loss. The results (Figure 4.2.4) clearly show that there is an inverse relationship between promastigote body size and its virulency status. In contrast, no changes were observed in this study in the flagellum length among the different passage numbers, although it was noted that the virulent parasites were highly motile in the culture, in comparison to the avirulent promastigotes. Interestingly, there was no difference in the growth curve patterns between virulent and avirulent parasites but there was a difference in the number of parasites at peak growth. Although the centrin gene was down regulated at passages 7 and 20, the parasite was still growing well (Figure 4.2.3). This was not in agreement with previous findings by

4) *L. mexicana* promastigote growth characterisation

Selvapandian, *et. al.*, (2001; 2004), who illustrated that the growth of *L. donovani* was highly disrupted as a result of centrin gene knockout. The difference could be explained by the adaptation time required for the parasite to grow under laboratory conditions. Another possibility is that, in this model when parasites were left to grow for long periods of time, this affected the overall gene expression profile. This may differ from the gene knockout studies where particular genes were deleted from the parasite. Since *in vitro* passage of *L. mexicana* has caused infectivity loss of the parasites, which was characterised by a dramatic delay in lesion appearance at the site of inoculation of Balb/c mice, the *Leishmania* parasites needed to be passaged regularly in laboratory animals to maintain their virulence status. Moreira, *et. al.*, (2012), showed similar findings, where a rapid loss of *L. infantum* virulence was reported when parasites were continuously grown in axenic culture, which consequently affected their capability to infect macrophages, meaning that they failed to infect susceptible mice.

It has been reported that *Leishmania* promastigotes enter the phagosomes of phagocytic cells such as DCs, neutrophils and macrophages. However, they can only multiply within macrophages, so these cells are considered to be the ideal host for the intracellular stage of *Leishmania* spp (Laufs, *et. al.*, 2002). The results of the current study have demonstrated that U937 monocytes were also able to host virulent, but not avirulent, *L. mexicana* promastigotes. In addition these parasites were able to produce the amastigote stage, so infectivity of *Leishmania* species in monocytes might be cell type and *Leishmania* spp dependent, and also most importantly on the virulence status of the parasites used.

In order to be able to survive and proliferate inside the acidic conditions of the phagosomes, *Leishmania* spp and other intracellular microorganisms have to suppress phagosome maturation, therefore these pathogens have developed survival mechanisms which are poorly understood (Burchmore and Barrett, 2001). The current study has demonstrated that avirulent *L. mexicana* promastigotes were unable to meet the two important survival requirements inside host cells. Firstly, the inability of avirulent parasites to produce the amastigote stage and proliferate inside infected U937 macrophages (Figure 4.2.9), compared to virulent parasite where amastigotes stage developed *in vitro* after 5 hours at 37°C as of infection target cells as illustrated in Figure 4.2.9A. There is no published data of a similar type of results. However, it has been reported that, in order to obtain *L. donovani* amastigotes, THP-1 cells have to be

4) *L. mexicana* promastigote growth characterisation

infected for 48 hours (De Muylder, *et. al.*, 2011). Secondly, the inability of avirulent *L. mexicana* promastigotes to delay or alter phagosome maturation, which was assessed by the high expression of matured phagosome markers Rab7 and Rab9, when compared to virulent parasites (Figure 4.2.13, A and B).

Phagosomes and lysosomes are important components in the control of pathogen infection. Inside the phagosomes, foreign antigens will be captured, and degraded by lysosomal enzymes. *Leishmania* parasites have developed many mechanisms to avoid being lysed and degraded by these enzymes at early stages of entering the host cell. The qPCR analysis demonstrated a significant down regulation of expression of these molecules in target cells infected with virulent (P 1) compared to avirulent (P 20) *L. mexicana*. This may enhance the killing of avirulent parasites by the phagosome–endosome fusion compared to virulent parasites. A study by Desjardins and Descoteaux (1997) using knockout LPG2 *L. donovani* illustrated the direct role of LPG2 in *L. donovani* promastigotes in phagosome–endosome fusion.

In this study, the expression of a phagosome maturation (which reflects parasite clearance due to phagosome-lysosome fusion) marker was used to address differences between virulent and avirulent *L. mexicana* promastigotes. The down regulation of Rab7 and Rab9 (apart from 48 hours post infection) in U937 macrophages infected with virulent parasites, may explain the virulent parasite's ability to transform to the amastigote stage, in comparison to avirulent parasites.

This study has also demonstrated the inability of amastigotes isolated from infected U937 macrophages infected with virulent parasites for 48 hours, to proliferate when cultured under the same conditions but in the absence of their target cells. This may emphasise the need for acidic conditions for amastigote proliferation, and is confirmed by the ability of *Leishmania* amastigotes to proliferate inside the acidic parasitophorous vacuole of infected cells. The pH also has an essential role in transformation of promastigotes into amastigotes.

In addition to the role of promastigote surface molecules such as GP63 and LPG, which facilitate promastigote-macrophage adhesion and intracellular survival respectively, the presence of glucose in culture media also affects infectivity in macrophages. It has been reported that *L. mexicana* expresses three isoforms of glucose transporter genes (LmGT), and the knockout of these genes in promastigotes reduced their ability to infect macrophages and thus prevented their differentiation into the amastigote stage

4) *L. mexicana* promastigote growth characterisation

(Burchmore, *et. al.*, 2003). In addition to the glucose, other monosaccharaides with six carbon atoms (hexoses), are required for amastigote survival, through their roles in the phosphate pathway that maintains parasite redox balance and generates precursors for DNA and RNA biosynthesis (Maugeri, *et. al.*, 2003). Another study by Naderer, *et. al.*, (2006) illustrated the ability of *L. major* to use an alternative pathway in the case of glucose deficiency inside the phagosomes. It was also reported that deletion of fructose-1, 6-bisphosphatase (FBP) enzyme in *L. major*, an enzyme responsible for gluconeogenesis (a metabolic pathway that results in the generation of glucose from non-carbohydrates) did not affect their ability to infect macrophages, and transformation to the amastigote stage, but did inhibit their ability to multiply.

The growth of avirulent *L. mexicana* promastigotes was inhibited, and the promastigotes were unable to survive and proliferate in conditioned medium derived from host cells infected with virulent or avirulent parasites. In contrast, this conditioned medium supported the growth of virulent parasites (Figure 4.2.11). These data shed more light on the importance of different soluble materials released by infected cells to control parasite growth, as reviewed in Ali, *et. al.*, (2013).

The current study has also demonstrated a significant increase in amastigote numbers at 24 hours of infection with virulent *L. mexicana*, without much decrease in host cell numbers. However, 48 hours of infection induced a significant decrease in target cell numbers, suggesting cell lysis. In addition, the expression of a specific amastigote protein A2 in infected target cells was significantly increased from a low level at 2 hours infection to reach its maximum expression at 24 hours. However, this expression was down regulated at 48 hours post infection. This seems to correlate well with the rate of infected cell lysis at 48 hours. This study has also demonstrated the inability of the amastigotes isolated from infected cells to proliferate, compared to the axenic amastigotes, which were generated in the laboratory (the late log growth phase of virulent and avirulent *L. mexicana* promastigotes cultured in Schneider *Drosophila* medium (Lonza, UK), supplemented with 20% v/v HIFCS and Hemin (Sigma, UK) at 32.5°C, 5% v/v CO₂). The results were in agreement with a study by Burchmore, *et. al.*, (2001), who reported that *Leishmania* amastigote proliferation was performed in the presence of acidic conditions inside an intracellular compartment called the parasitophorous vacuole, and that the pH also had an essential role in transformation of promastigotes into amastigotes.

4) *L. mexicana* promastigote growth characterisation

Although the number of avirulent promastigotes was considerably less at the three tested time points, 48 hours infection clearly showed a high number of promastigotes per macrophage, which failed to progress to the amastigote stage (Figure 4.2.11). These results indicate an important fact, that the parasite's infectivity should be measured not only by infection of the target cells with the promastigotes, but also most importantly by the successful amastigote stage performance inside infected macrophages.

In conclusion, this model has produced virulent and avirulent *L. mexicana* promastigotes. The loss of virulency was characterised by *in vivo* failure to infect susceptible Balb/c mice, and also *in vitro* inability of avirulent parasites to transform into the pathogenic amastigote stage, which can survive and proliferate inside susceptible human cell lines. The methods used in this study to demonstrate mechanisms of virulency loss in *Leishmania* could be applied to other parasite species to produce attenuated forms that might be used as vaccine candidates.

**Chapter 5/ MHC class I expression following
L. mexicana promastigote infection**

5. MHC class I expression following *L. mexicana* promastigote infection

5.1 Introduction

Antigen presenting cells (APCs) have an important role in immune responses against infection, through the processing of foreign antigens and presentation *via* MHC class I and MHC class II. Nevertheless, many intracellular pathogens, like those that cause tuberculosis, Leishmaniasis, and Chagas' disease have developed specific strategies to survive inside macrophages to avoid the immune response (Desjardins and Descoteaux, 1998; Overath and Aebischer, 1999). Consequently, the ability of pathogen-infected APCs to activate T cells response through antigen presentation is reduced (Alexander and Russell, 1992; Alexander, *et. al.*, 1999).

Leishmania parasites as an intracellular pathogen can successfully hide and protect themselves by being inside APCs such as macrophages and DCs. Interestingly, even inside the APCs these organisms have an extra protection by being in small compartments called parasitophorous vacuoles inside host APCs, in particular macrophages (Prina, *et. al.*, 1990; Russell, *et. al.*, 1992). The presence of PVs inside the infected APCs has been shown in some studies not to affect cell surface expression of the MHC class I and MHC class II, however only MHC class II but not MHC class I molecules were detected on the PVs surfaces (Olivier, *et. al.*, 2005). The mechanisms by which the *Leishmania* parasite manipulates antigen presentation *via* MHC molecules is poorly understood. Variable data have been reported on *Leishmania* antigen presentation, which seems to be parasite species and host cell dependent. It has been reported that murine macrophages infected with the metacyclic promastigotes of *L. major* or *L. amazonensis* present very low LACK antigen. However, when they are infected with the amastigote stage of these parasites, they present no parasite antigen at all (Courret, *et. al.*, 1999). This illustrated promastigote susceptibility and amastigote resistance to the host cell's defense mechanisms.

Both *L. amazonensis* amastigote infected and non-infected bone marrow macrophages from Balb/c mice and C57BL/6 mice have been found to express more MHC class I on the cell surface, and MHC class II, was mainly found to be associated with the PV membranes (Lang, *et. al.*, 1994).

5) MHC class I expression following *L. mexicana* infection

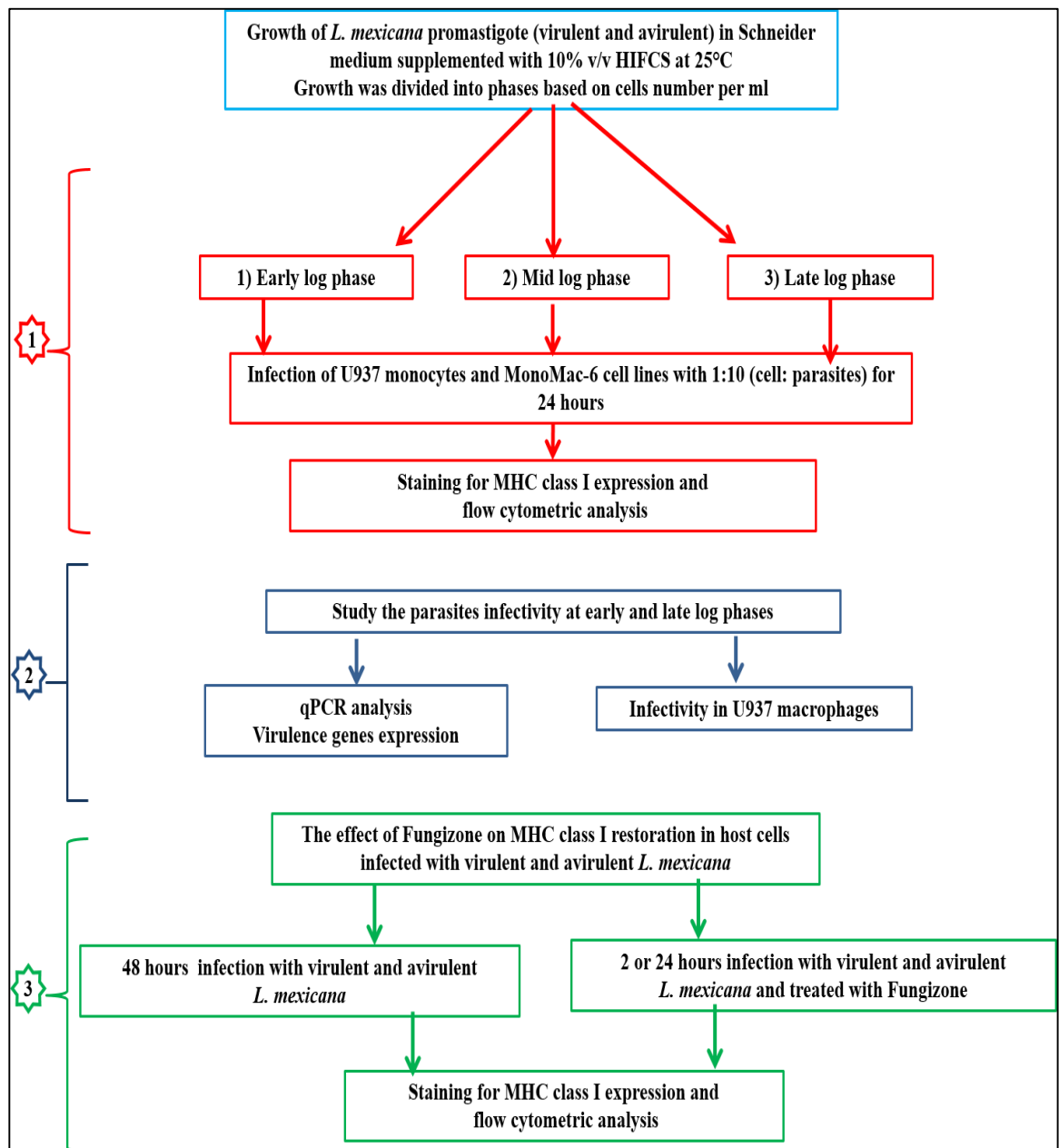
A recent study reported that *L. donovani* caused a down regulation of MHC class I and MHC class II through the effect on phagolysosome (the combination of phagosomes and lysosomes) biogenesis. This was due to the presence of GP63, which affect processing of degraded parasites inside the PVs, and presentation on MHC class I or class II molecules to T cells (Matheou, *et. al.*, 2013).

Although macrophages are the main host for *Leishmania* species, other cells such as DCs and monocytes, which take part in an anti-Leishmanial immune response, can also act as *Leishmania* spp hosts (Gorak, *et. al.*, 1998). A number of studies have investigated the antigen presentation potential of *Leishmania*-infected APCs, but results have been contradictory. For example, a study by Kima, *et. al.*, (1997) reported that a specific CD8 cell line was generated against GP46/M-2, a *L. amazonensis* antigen. In further experiments, those CD8 cells were shown to recognize mammalian cells transfected with plasmid encoding GP46/M-2, as well as macrophages infected with *L. amazonensis*, which presented GP46/M-2 on their MHC class I molecules. The activation of CD8 was eliminated after treatment of the infected cells with brefeldin A to block antigen presentation, and this finding revealed the role of MHC class I in *Leishmania* infection control. In another study by Meier, *et. al.*, (2003), infection with *Leishmania* parasites inhibited T cell activation, which was not related to MHC class II expression on the surface of infected macrophages. It was also demonstrated that infection with *L. donovani* decreased the macrophages ability to present parasite antigens (Sundar, *et. al.*, 1997). Since the MHC class I molecule is a key factor in parasite recognition and elimination by the immune system, this study has investigated the effect of infection of host cells with *L. mexicana* promastigotes on MHC class I expression. Due to the significant role of monocytes in the entry of microorganisms, including *Leishmania* spp into host cells, two monocyte cell lines (U937 and MonoMac-6) in addition to U937 macrophages were used to investigate the effect of three important variables: virulence states, growth phases of the parasite, and the infection load, on the expression of MHC class I in susceptible human cell lines.

5) MHC class I expression following *L. mexicana* infection

The work flow

The experiments of this chapter were carried out according to the following schedule:



5.2 Results

5.2.1 *In vitro* growth of *L. mexicana* promastigotes

The *L. mexicana* promastigote growth curve was divided into three stages, based on their density in the culture (parasites per ml) and these stages were used to study MHC class I expression in infected host cells. There was a gradual increase in parasite number during the first few days in culture (early log phase $\geq 5 \times 10^6$ promastigotes per ml), which was followed by a steep increase in the density of the parasite number per ml (mid log stage and $\geq 1 \times 10^7$ per ml). A maximum *L. mexicana* promastigote growth in culture was identified at the late log phase, where the number of parasites reached 2.5×10^7 per ml or above. There was a sharp decrease in cell number when the parasite reached the peak of growth (Figure 5.2.1.A). Results in this study show that the range of the growth curve was dependent on the initial number of parasites in the inoculum that was used to initiate the growth curve cultures. For instance, the growth curve period lasted 10 and 25 days when cultures were started with a concentration of 1×10^6 per ml (Appendix 13), and 1×10^5 (Figure 5.2-1), respectively. The results clearly show that there was no stationary phase (horizontal line) in the *L. mexicana* growth curve under anaerobic conditions, which used in this study. The stationary phase in this study were recognized by parasite number (where the population peaked) in the culture according to Mottram, 2008 (personal communication), and a slight modification to the original protocol as presented in Figure 5.2.1.B.

5) MHC class I expression following *L. mexicana* infection

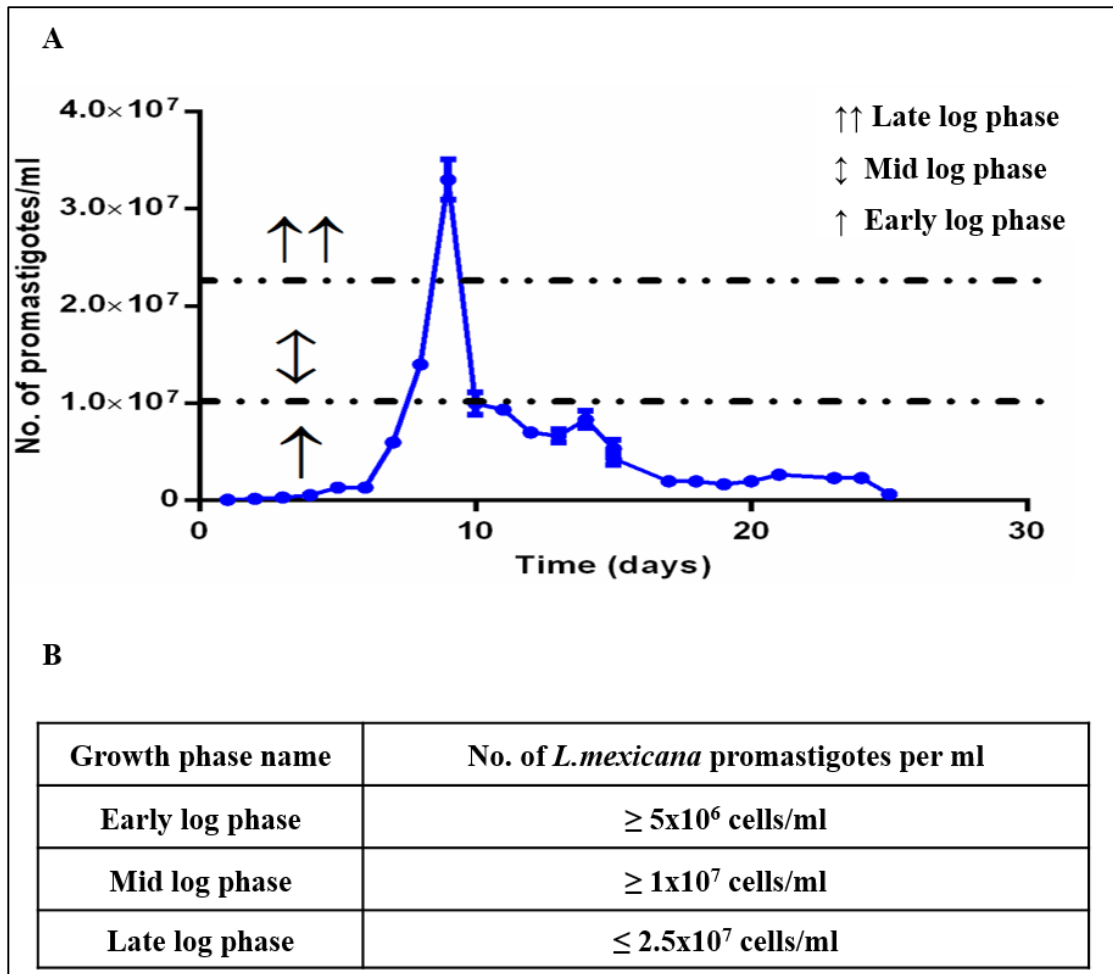


Figure 5.2-1 *In vitro* growth curve of *L. mexicana* promastigotes

(A) Fresh cultures of *L. mexicana* promastigotes (passage 1) were initiated at a concentration of 1×10^5 promastigotes per ml in culture medium. Parasite samples (10 μ l) were fixed in 2% w/v paraformaldehyde and counted daily. The graph represents 3 independent experiments. Data are presented as mean + SEM. The growth curve of *L. mexicana* promastigotes in this study was divided into three different phases according to the number of promastigotes per ml as is shown in the Table (B). The early, mid and late log growth phases were used to investigate the MHC class I expression in infected host cells (U937 monocyte and MonoMac-6 cells) with *L. mexicana* promastigotes.

5) MHC class I expression following *L. mexicana* infection

5.2.2 Effect of *L. mexicana* promastigote infection on MHC class I expression in susceptible human cell lines

In this study the effect of infection with *L. mexicana* promastigotes on the expression of MHC class I was investigated using susceptible human U937 monocyte and MonoMac-6 monocytic cell lines. Target cells cultured in 24 well plates at a concentration of 2×10^5 cells per ml per well were infected with different ratios of *L. mexicana* promastigotes taken at different points of the growth curve for 24 hours at 37°C in a 5% v/v CO₂ atmosphere.

5.2.2.1 Effect of infection with early growth phase of *L. mexicana* promastigotes on MHC class I expression

Results clearly show that infection with avirulent and virulent *L. mexicana* promastigotes reduced the proportion of infected cells that expressed MHC class I, from 92.62% of non-infected U937 monocytes, to 33.41% and 29.84% of cells that have been infected with virulent and avirulent parasites, respectively. Meanwhile, the infection of MonoMac-6 cells with avirulent and virulent *L. mexicana* reduced the proportion of cells expressing MHC class I expression from 78.48% of control cells to 37.14% and 45.51% respectively (Figure 5.2.2). Further analysis of the median fluorescence intensity (MFI) revealed similar patterns of effects to those that were observed by analysing the percentage of MHC class expression.

5) MHC class I expression following *L. mexicana* infection

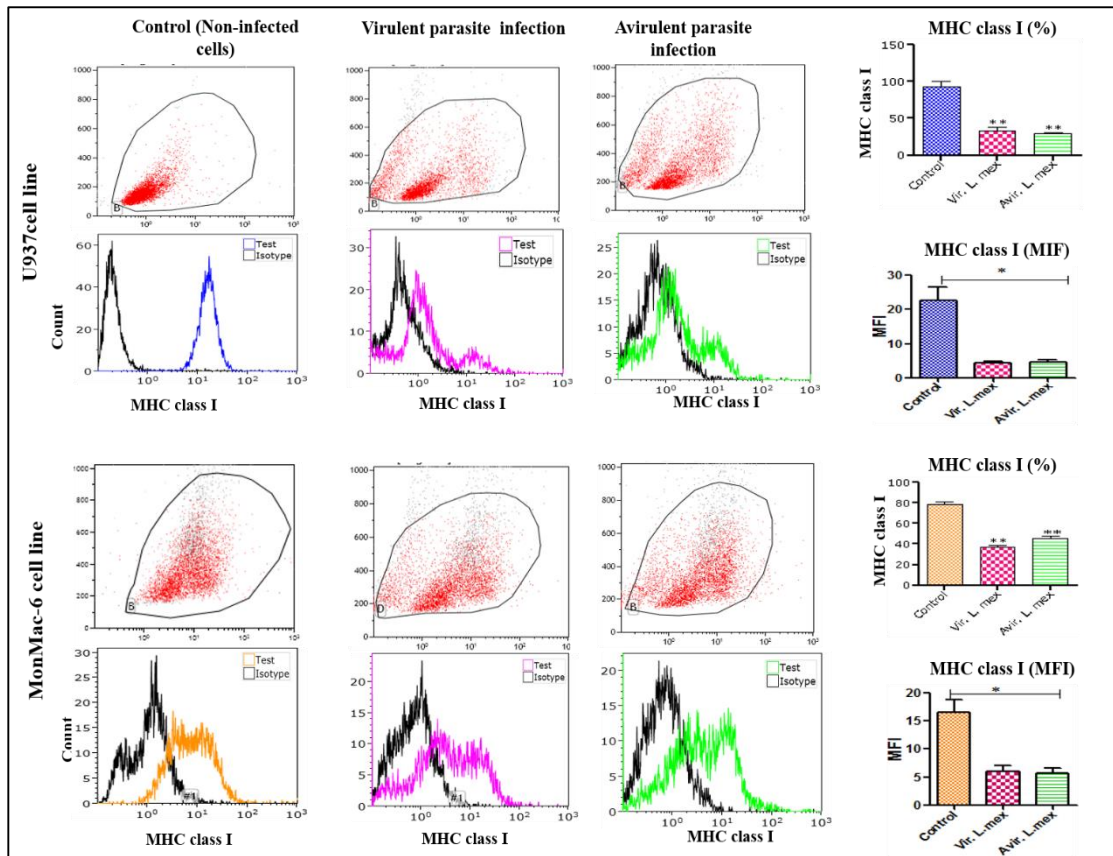


Figure 5.2-2 Effect of infection with early log *L. mexicana* promastigotes on MHC class I expression.

Flow cytometric histograms illustrating MHC class I expression in non-infected (control) and infected U937 monocyte (top panel) and MonoMac-6 cell line (lower panel). Target cells were seeded in 24 well plates at a concentration of 2×10^5 cells per well, and infected with virulent and avirulent early log phase *L. mexicana* promastigotes at a ratio of 1:10 (cell: parasite) for 24 hours at 37°C. Cells were fixed with 2% w/v paraformaldehyde and stained for MHC class I expression. Data are derived from at least 3 independent experiments for each cell line* P<0.05, ** P<0.01 using the Student's t-test. Data are presented as mean + SEM.

5) MHC class I expression following *L. mexicana* infection

5.2.2.2 Effect of infection with mid log phase of *L. mexicana* promastigotes on MHC class I expression

Interestingly, although the parasite at mid log phase were supposed to be more mature compared to the previous phase, their capacity to reduce the proportion of infected cells expressing MHC class I following infection was reduced. Results showed that 92.52% of non-infected U937 monocytes expressed MHC class I expression, whereas only 80.76% and 79.50% of cells infected with virulent and avirulent parasites expressed MHC class I respectively (Figure 5.2.3, top panel). Similarly, infection with the mid log phase had a minimal effect on the proportion of MonoMac-6 cells expressing MHC class I (80.72% of control cells, but 69.69% and 68.63% of cells following the infection with virulent and avirulent parasites respectively, Figure 5.2.3, lower panel). Although the analysis of the percentage of MHC class I positive cells did not show a difference in MHC class I expression between control and infected cells, the MFI analysis reveals a significant down regulation of MHC class I expression following infection of U937 monocyte, but not following infection of MonoMac-6 cells. The down regulation of MHC class I expression was more pronounced following infection with avirulent parasites.

5) MHC class I expression following *L. mexicana* infection

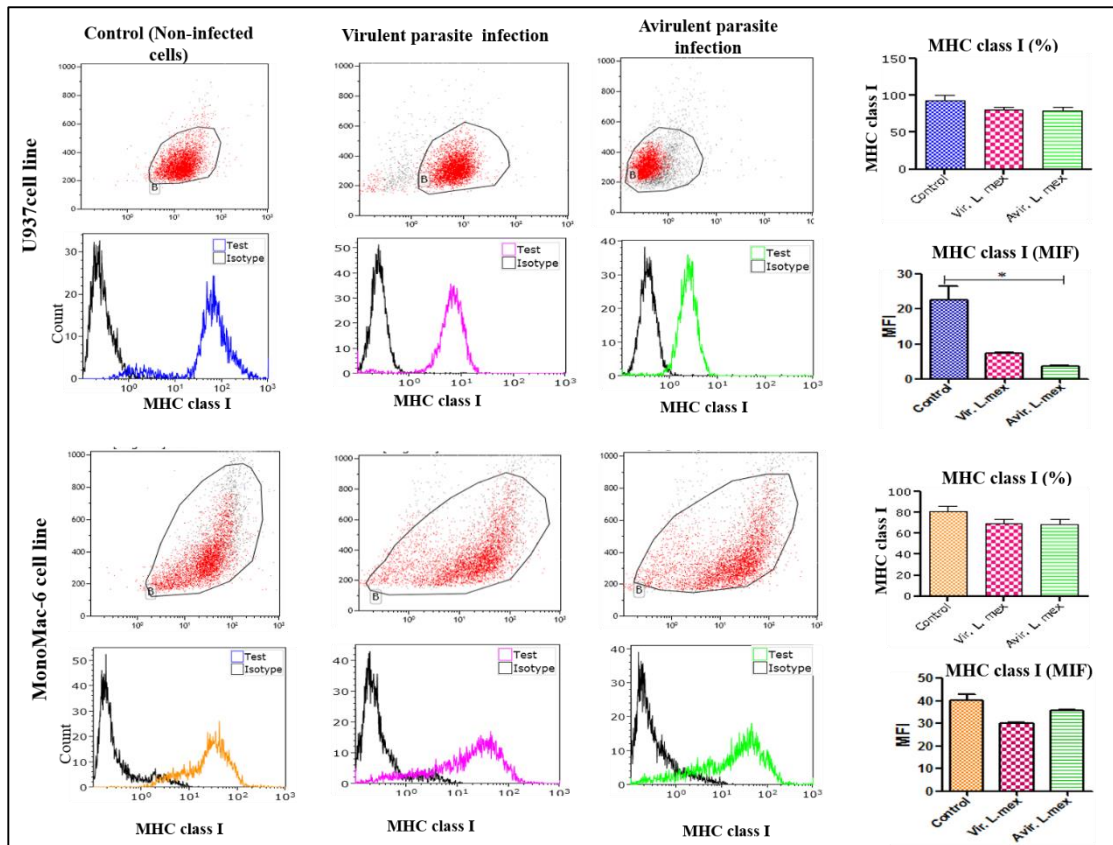


Figure 5.2-3 Effect of infection with mid log *L. mexicana* promastigotes on MHC class I expression.

Flow cytometric histograms illustrating MHC class I expression in non-infected (control) and infected U937 monocyte (top panel) and MonoMac-6 cell line (lower panel). Target cells were seeded in 24 well plates at a concentration of 2×10^5 cells per well, and infected with virulent and avirulent mid log phase *L. mexicana* promastigotes at a ratio of 1:10 (cell: parasite) for 24 hours at 37°C. Cells were fixed with 2% w/v paraformaldehyde and stained for MHC class I expression. Data are derived from at least 3 independent experiments for each cell line, * $P < 0.05$, using the Student's t-test. Data are presented as mean + SEM.

5) MHC class I expression following *L. mexicana* infection

5.2.2.3 Effect of infection with late log phase of *L. mexicana* promastigote on MHC class I expression

Infection of host cells with either late log phase virulent or avirulent *L. mexicana* promastigotes at a ratio of 1:10 (cell: parasites) had a limited effect on the proportion of cells expressing MHC class I. 88.94% of control U937 monocytes expressed MHC class I, compared to 77.98%, and 78.51% of cells infected with virulent and avirulent parasites, respectively. Similarly, 77.92% of control MonoMac-6 cells expressed MHC class I, compared to 54.26%, and 53.1% of cells following infection with virulent or avirulent *L. mexicana* (Figure 5.2.4). Infection had no influence on the intensity of MHC class I expression on U937 monocytes, whereas the intensity of MHC class I expression on the MonoMac-6 cell line was significantly down regulated following infection with virulent (but not avirulent) *L. mexicana* promastigotes.

5) MHC class I expression following *L. mexicana* infection

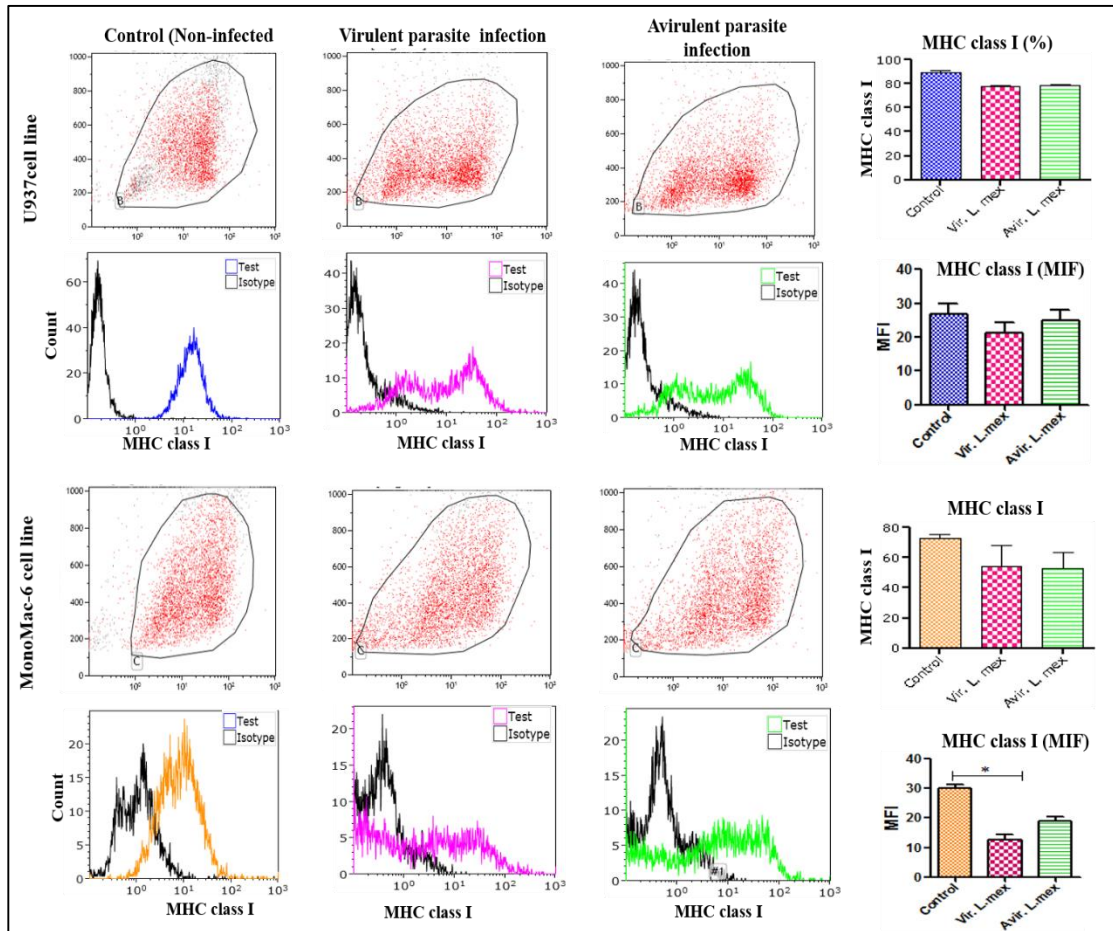


Figure 5.2-4 Effect of infection with late log *L. mexicana* promastigotes on MHC class I expression.

Flow cytometric histograms illustrating MHC class I expression in non-infected (control) and infected U937 monocyte (top panel) and MonoMac-6 cell line (lower panel). Target cells were seeded in 24 well plates at a concentration of 2×10^5 cells per well, and infected with virulent and avirulent late log phase *L. mexicana* promastigotes at a ratio of 1:10 (cell: parasite) for 24 hours at 37°C. Cells were fixed with 2% w/v paraformaldehyde and stained for MHC class I expression. Data are derived from at least 3 independent experiments for each cell line, * $P < 0.05$, using the Student's t-test. Data are presented as mean + SEM.

5.2.3 Analysis of *L. mexicana* promastigote infectivity at early and late log growth phases

The unexpected finding of down regulation MHC class I expression in target cells (U937 and MonoMac-6 monocytes), induced by early log phase infection compared to mid and late log phases, has raised the question as to whether those findings were related to changes in the infectivity of *L. mexicana* promastigotes at different growth phases in culture. Therefore, total mRNA was extracted from both early and late log phases using the Qiagen kit. RNA was converted to cDNA, which used for qPCR analysis to determine the virulence associated gene expression in early and late log phases of virulent *L. mexicana* promastigote growth. The results in (Figure 5.2.5.A) clearly show that the expression of GP63, LPG2, Cht1, CPC, CPC2 and CPC2.8 genes was significantly higher ($P>0.05$) in the late log phase compared to early log phase.

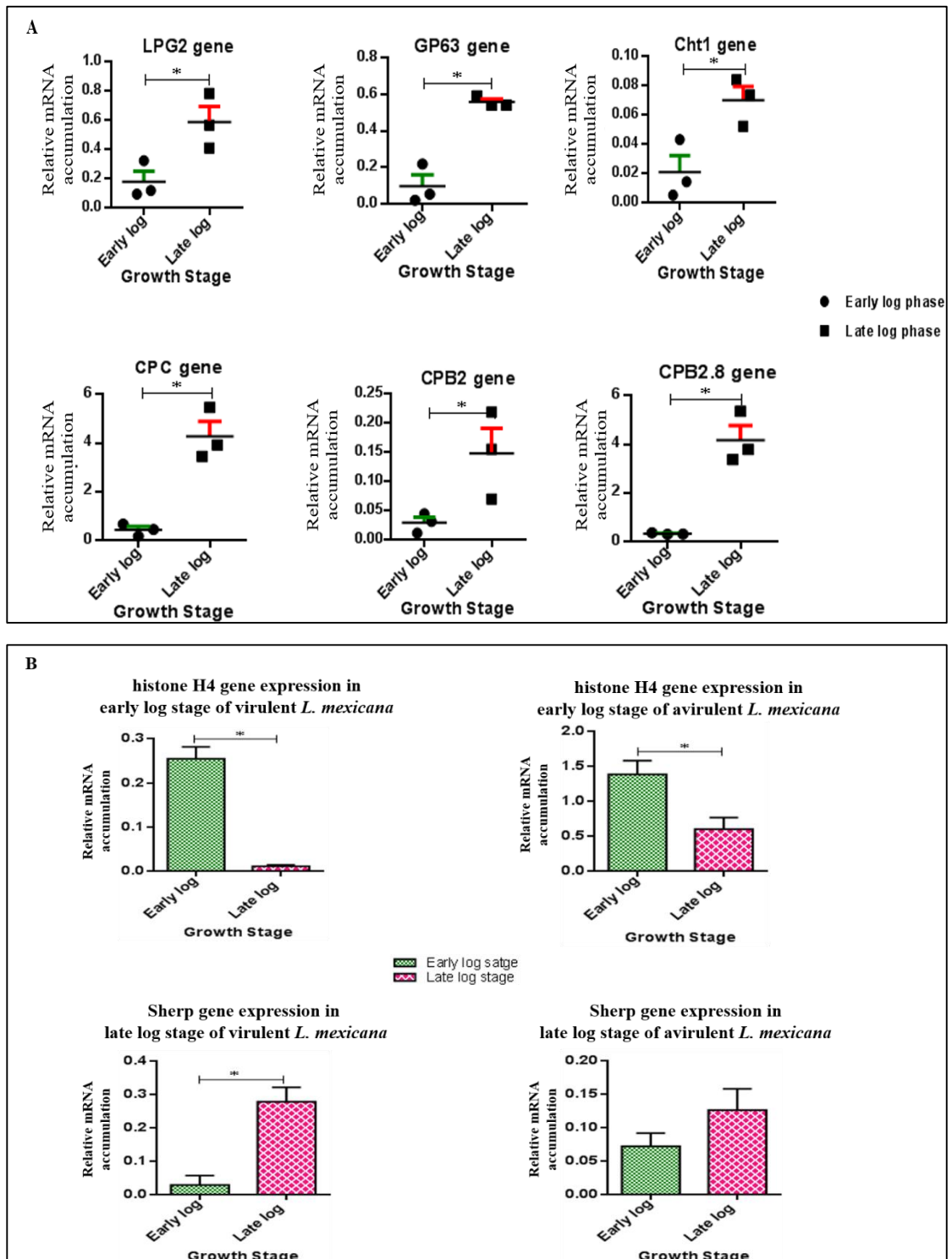
Moreover, parasite infectivity at the log phase (metacyclic) was further confirmed by analysis of the expression of Small Hydrophilic Endoplasmic Reticulum-associated Protein-Sherp (accession number XM-003875691) and histone H4 (accession number XM-003879078) genes which have been used to evaluate metacyclogenesis in promastigotes (Moreira, *et. al.*, 2012). mRNA was extracted from the early and late log stage of virulent (P1) and avirulent (P20) *L. mexicana* cultures. One microgram of each RNA sample was converted into cDNA, which was then used for qPCR analysis. The results (Figure 5.2.5.B) clearly show histone H4 was highly expressed in the early log (exponential) phase compared to late log (stationary) phase, while the Sherp gene was significantly expressed more in the late (stationary) phase of the virulent parasite compared to the early (exponential) phase. Similarly, the Sherp gene was expressed more in the late log stage of the avirulent parasite compared to the early log phase, but the difference was not significant.

In addition to the above results, the infectivity of *L. mexicana* during early and late log phases was also confirmed by infection of target cells with both growth stages of the parasites. The results showed the failure of *L. mexicana* promastigotes at early log stage to produce the amastigote stage in infected host cells (Figure 5.2.5.C).

In addition, Giemsa staining illustrated that more U937 monocytes and macrophages were infected using the parasite at the late log stage compared to the number of infected cells using the parasite at early log stage (data not shown). Taking all those results

5) MHC class I expression following *L. mexicana* infection

together, *L. mexicana* promastigotes at the late log phase was considered as being the infectious stage, which was further investigated in this study.



5) MHC class I expression following *L. mexicana* infection

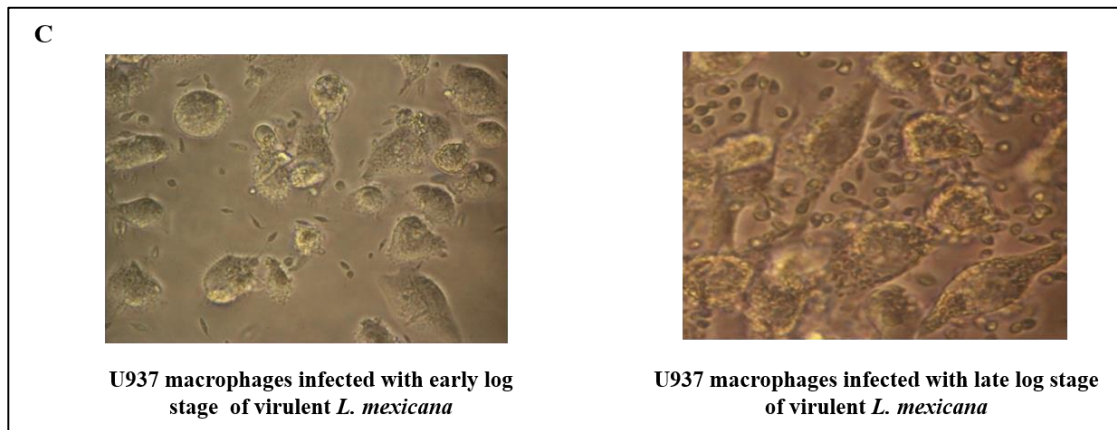


Figure 5.2-5 Gene expression profile and infectivity *L. mexicana* promastigotes at early and late log phases

(A) The expression of virulence associated genes in the early and late log growth phases. mRNA was extracted from promastigotes at early and late log growth phases of promastigotes, and converted to cDNA to be used as DNA template for qPCR reactions. (B) the expression of *Serp* and *H4* genes in early and late log growth phases of virulent and avirulent *L. mexicana* promastigotes. Results were normalized with *L. mexicana*- β tubulin and *L. mexicana* α -tubulin housekeeping genes. Data are derived from 3 independent experiments. * $P < 0.05$ using the Mann–Whitney U test. Data are presented as mean + SEM. (C) U937 macrophages infected with virulent *L. mexicana* promastigotes at early (Left), and late log phase (Right). Target cells were infected with both stages of growth at a ratio of 1:10 (cell: parasites) for 24 hours at 37°C, and infected cells were monitored and visualized for production of amastigote stages.

5) MHC class I expression following *L. mexicana* infection

5.2.4 Effect of infection with late log *L. mexicana* promastigotes on MHC class I expression using 1:20 ratio

Increasing the dose of infection from 1:10 to 1:20 (cell: parasites) significantly reduced the proportion of U937 monocytes and MonoMac-6 cell lines expressing MHC class I. Unexpectedly, infection with avirulent *L. mexicana* promastigotes had a more pronounced effect than infection with virulent parasites in both cell lines. Results showed that infection with virulent and avirulent parasites reduced the proportion of U937 monocytes expressing MHC class I from 91.75% to 60.07%, and 34.18% respectively. In contrast, infection with virulent and avirulent *L. mexicana* promastigotes reduced the proportion of MonoMac-6 cells expressing MHC class I molecules from 80% in non-infected to 53.78% and 24.27% respectively (Figure 5.2.6). Similar results were obtained when the intensity of MHC class I expression was examined (on the basis of the MFI) on U937 and MonoMac-6 cells following the parasite infection. Infection significantly down regulated MHC class I expression, and the magnitude of this effect was more pronounced following infection with avirulent parasites.

5) MHC class I expression following *L. mexicana* infection

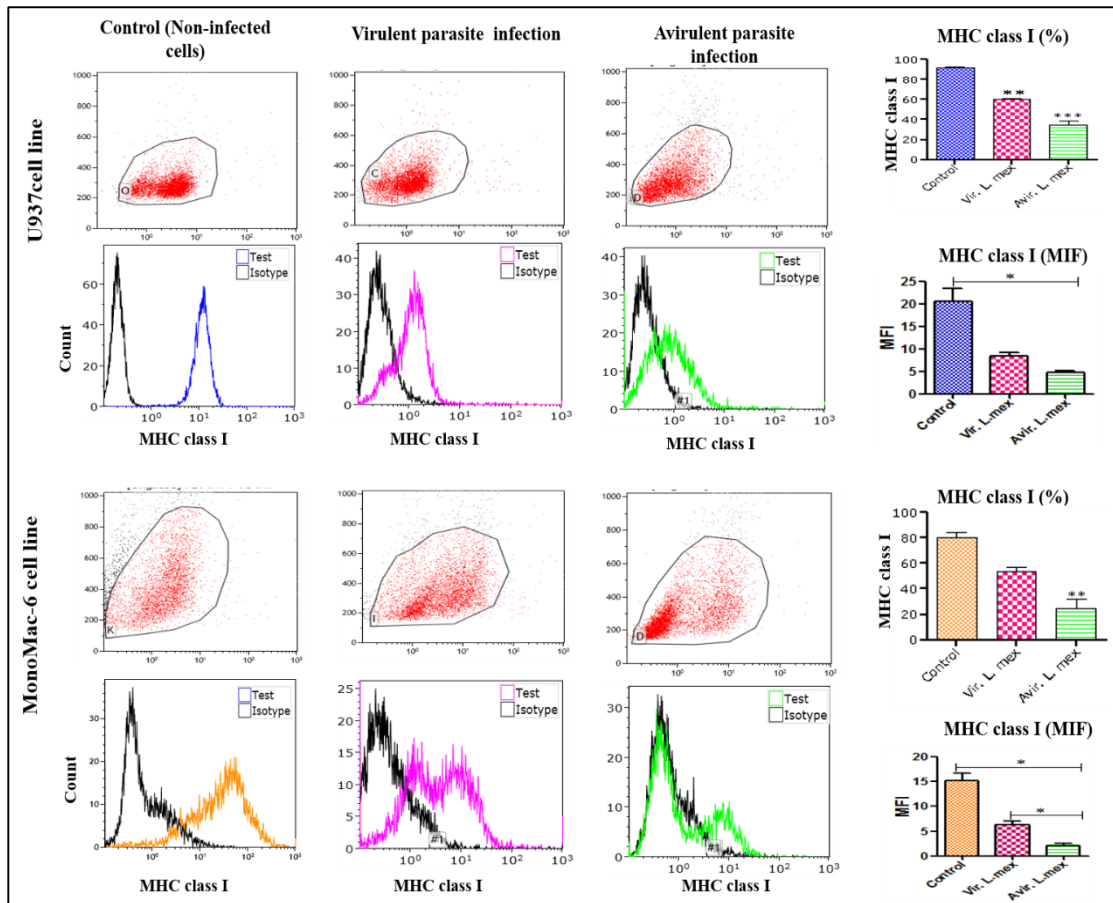


Figure 5.2-6 Effect of infection with late log *L. mexicana* promastigotes on MHC class I expression using 1:20 ratio

Flow cytometric histograms illustrating MHC class I expression in non-infected (control) and infected U937 monocyte (top panel) and MonoMac-6 cell line (lower panel). Target cells were seeded in 24 well plates at a concentration of 2×10^5 cells per well, and infected with virulent and avirulent late log phase *L. mexicana* promastigotes at a ratio of 1:20 (cell: parasite) for 24 hours at 37°C. Cells were fixed with 2% w/v paraformaldehyde and stained for MHC class I expression. Data are derived from at least 3 independent experiments for each cell line, *P<0.05, **P<0.01 using the Student's t-test. Data are presented as mean + SEM.

5) MHC class I expression following *L. mexicana* infection

5.2.5 Effect of infection with high doses of late log phase of *L. mexicana* promastigotes on MHC class I expression

In order to investigate the effect of high infection doses using virulent and avirulent *L. mexicana* promastigotes at late log phase on the proportion of cells expressing MHC class I, U937 monocytes were infected with three doses of 1:30, 1:50 and 1:70 (cell: parasites). After 24 hours infection, cells were stained for MHC class I expression and analysed by flow cytometry. Results showed complete down regulation of MHC class I expression by U937 monocytes infected with the three tested doses (Figure 5.2.7).

5) MHC class I expression following *L. mexicana* infection

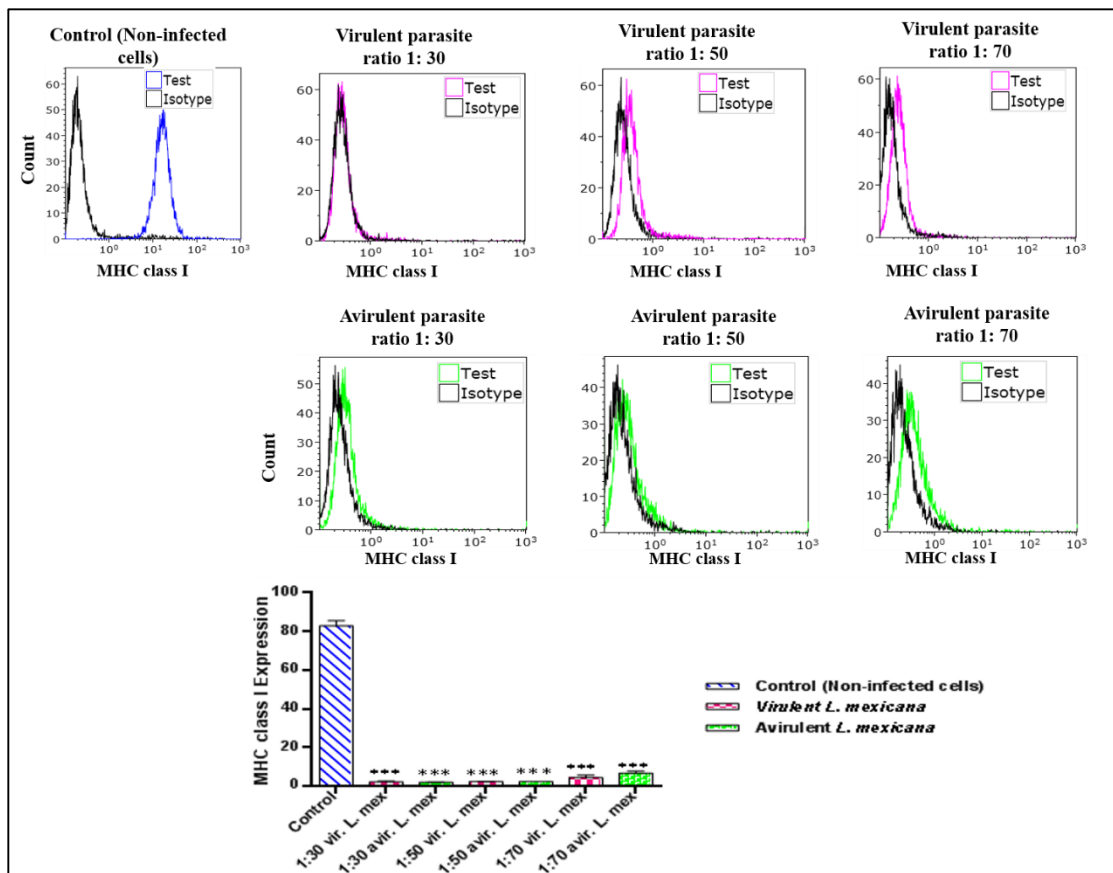


Figure 5.2-7 Effect of infection with high doses of late log phase of *L. mexicana* promastigotes on MHC class I expression

Flow cytometric histograms illustrating MHC class I expression in non-infected (control) and infected U937 monocytes. Target cells were seeded in 24 well plates at a concentration of 2×10^5 cells per well, and infected with late log phase of virulent and avirulent *L. mexicana* promastigotes at a ratios of 1:30, 1:50, and 1:70 (cell: parasite) for 24 hours at 37°C. Cells were fixed with 2% w/v paraformaldehyde and stained for MHC class I expression. Data are derived from at least 3 independent experiments for each cell line. *** $P < 0.001$, using the Student's t-test. Data are presented as mean + SEM.

5.2.6 Effect of Fungizone on MHC class I expression restoration in *L. mexicana* infected U937 monocytes

It was unclear whether MHC class I down regulation was reversible when the infection was eliminated by treatment with an anti-*Leishmania* agent. Fungizone at a concentration of 4µg per ml was optimal to kill *Leishmania* parasites, and also was not toxic to the target cells, as shown by MTT assay (data not shown). The results clearly show that short-duration (2 hours) infection of U937 monocytes with virulent and avirulent *L. mexicana* promastigotes significantly down regulated the expression of MHC class I molecules. However, treatment of infected cells with Fungizone 2 hours after infection resulted in a restoration of MHC class I expression (Figure 5.2.8.A).

The same pattern of results was obtained when U937 monocytes were infected for longer times (24 hours) and treated with Fungizone (Figure 5.2.8.B). In this experiment, target cells were infected with the virulent and avirulent parasites and treated with Fungizone for up to 24 hours. Interestingly, the results show that the level of restoration of MHC class I expression in cells infected with avirulent *L. mexicana* promastigotes was significantly higher compared to the restoration of MHC class I in cells infected with virulent promastigotes.

5) MHC class I expression following *L. mexicana* infection

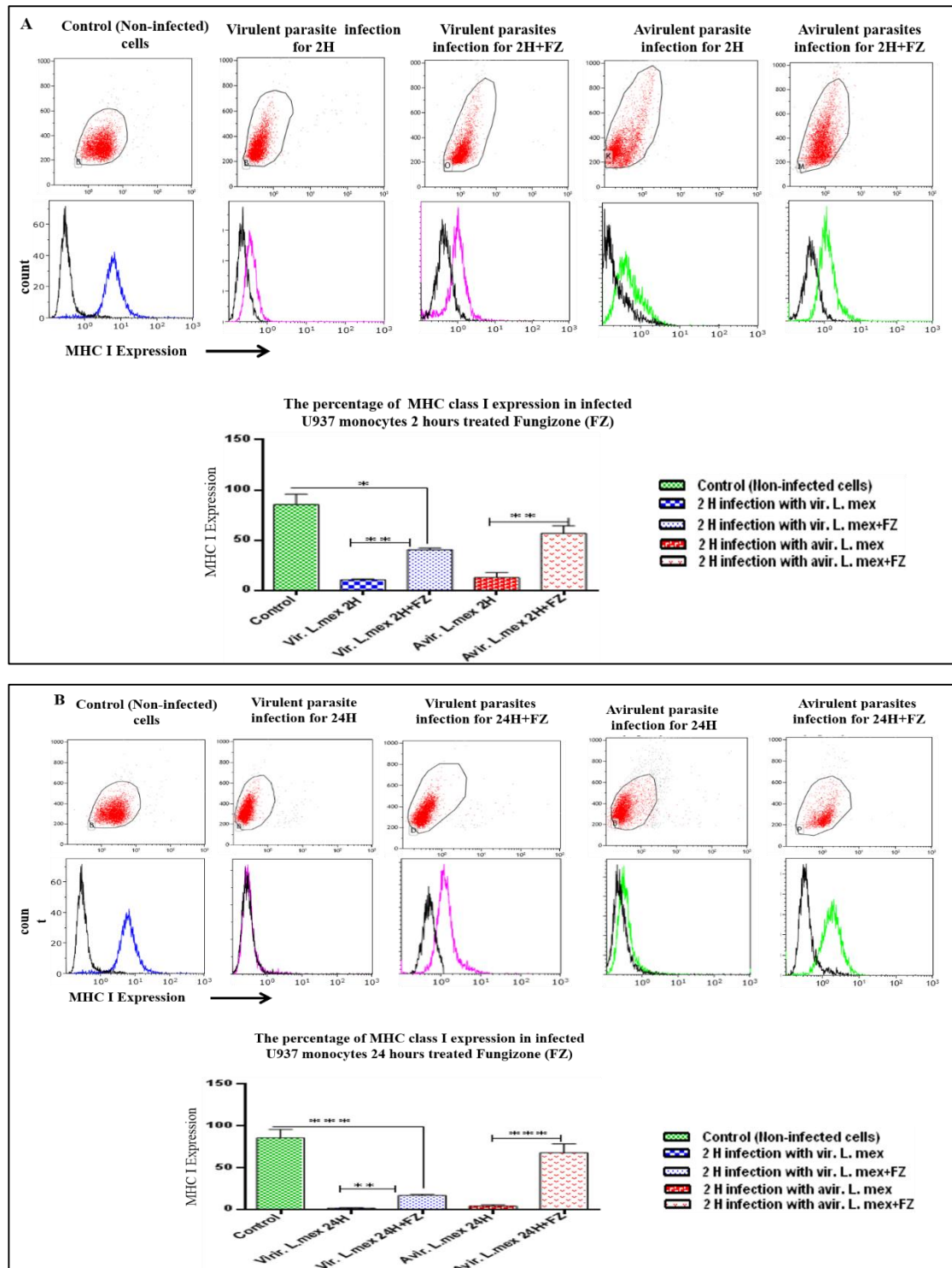


Figure 5.2-8 Effect of Fungizone on restoration of MHC class I expression in infected U937 monocytes

Target cells were infected at a ratio of 1:30 (cell: parasites), at different time points when cells were treated with 4 µg per ml Fungizone. **A** and **B** Flow cytometric histograms illustrate MHC class I expression in non-infected (control) and U937 monocytes infected with virulent at avirulent parasites for 2 and 24 hours, and then treated with Fungizone after 2 and 24 hours post-infection, respectively. Cells were fixed with 2% w/v paraformaldehyde and stained for MHC class I expression. Data are derived from at least 2 independent experiments for each cell line, *P<0.05, ** P<0.01, *** P<0.001 using the Student's t-test. Data are presented as mean + SEM.

5) MHC class I expression following *L. mexicana* infection

5.2.7 Effect of Fungizone on MHC class I expression restoration in U937 macrophages infected with *L. mexicana* promastigotes

In general, the effect of infection of U937 macrophages with virulent and avirulent *L. mexicana* promastigotes on MHC class I expression was similar to that of U937 monocytes, but with some differences. While the infection of U937 monocytes with virulent and avirulent parasite caused almost complete down regulation of MHC class I, this was not the case with U937 macrophages. Here, treatment of infected cells with Fungizone two hours after infection caused restoration of MHC class I expression (Figure 5.2.9.A) and treatment of U937 macrophages with Fungizone after 24 hours post infection resulted in a significant MHC class I expression (Figure 5.2.9.B).

5) MHC class I expression following *L. mexicana* infection

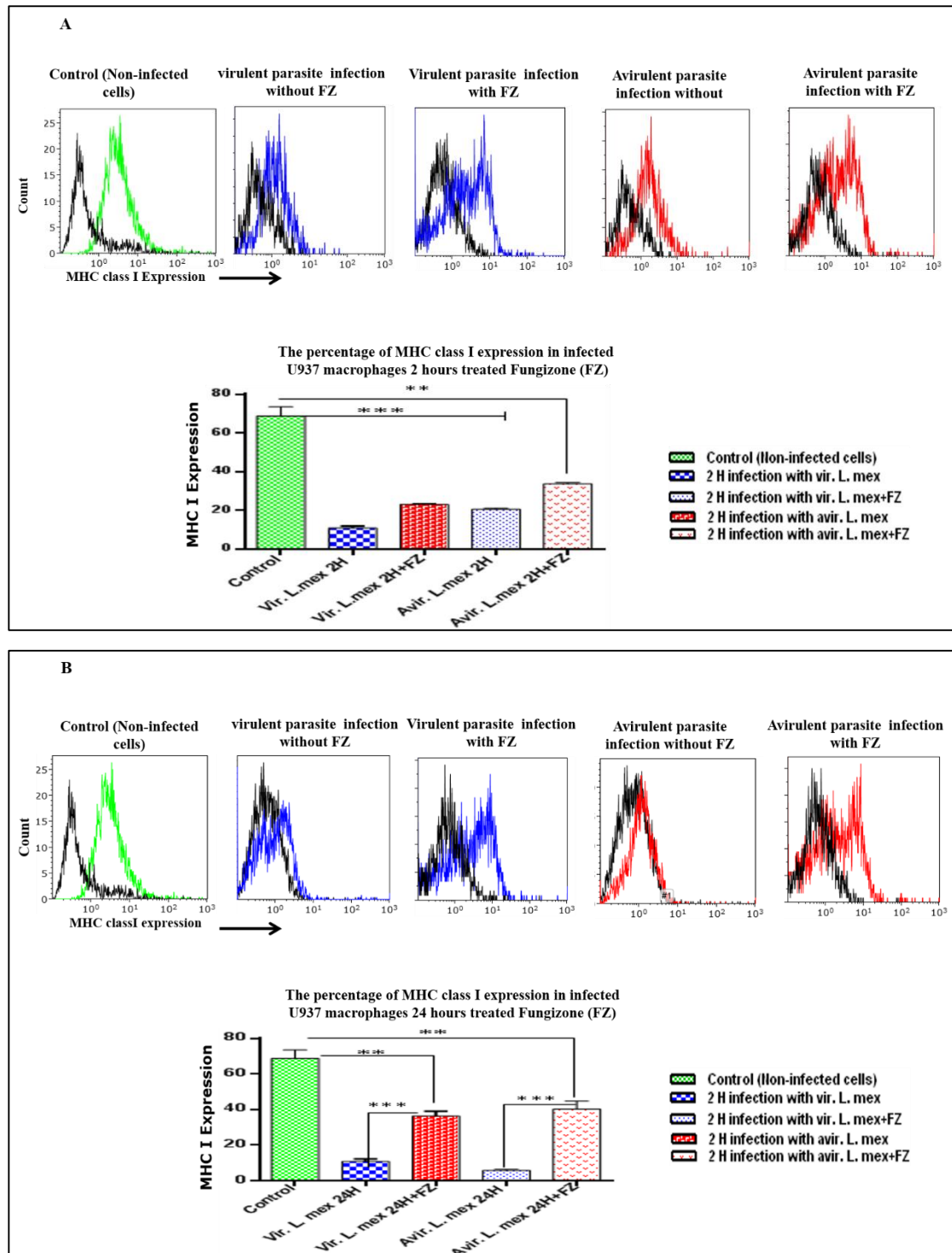


Figure 5.2-9 Effect of Fungizone on restoration of MHC class I in expression infected U937 macrophages

Target cells were infected with *L. mexicana* promastigotes at a ratio of 1:30 (cell: parasites), after different time points infected cells were treated with 4µg per ml Fungizone. **A** and **B** Flow cytometric histograms illustrate MHC class I expression in non-infected (control) and infected U937 macrophages with virulent and avirulent parasites for 2 and 24 hours, and then treated with Fungizone after 2 and 24 hours post infection, respectively. Cells were fixed with 2% w/v paraformaldehyde and stained for MHC class I expression. Data are derived from at least 2 independent experiments for each cell line. **P<0.01, ***P<0.001 using the Student's t-test. Data are presented as mean + SEM.

5.2.1 Spontaneous restoration of MHC class I expression in host cells infected with *L. mexicana* promastigotes

Further experiments have shown that MHC class I expression has been partially restored in U937 monocytes infected with virulent and avirulent *L. mexicana* promastigotes for 48 hours. This has happened spontaneously without treatment with Fungizone. The recovery of MHC class I expression was observed in both U937 monocytes infected with virulent and avirulent parasites, however, the recovery of MHC class I expression was greater in cells infected with avirulent compared to cells infected with virulent *L. mexicana*, as illustrated in (Figure 5.2.10. A).

In U937 macrophages infected with virulent *L. mexicana* for 48 hours, there was no restoration of MHC class I expression, compared to the significant MHC class I recovery in U937 macrophages infected with avirulent parasite (Figure 5.2.10.B).

5) MHC class I expression following *L. mexicana* infection

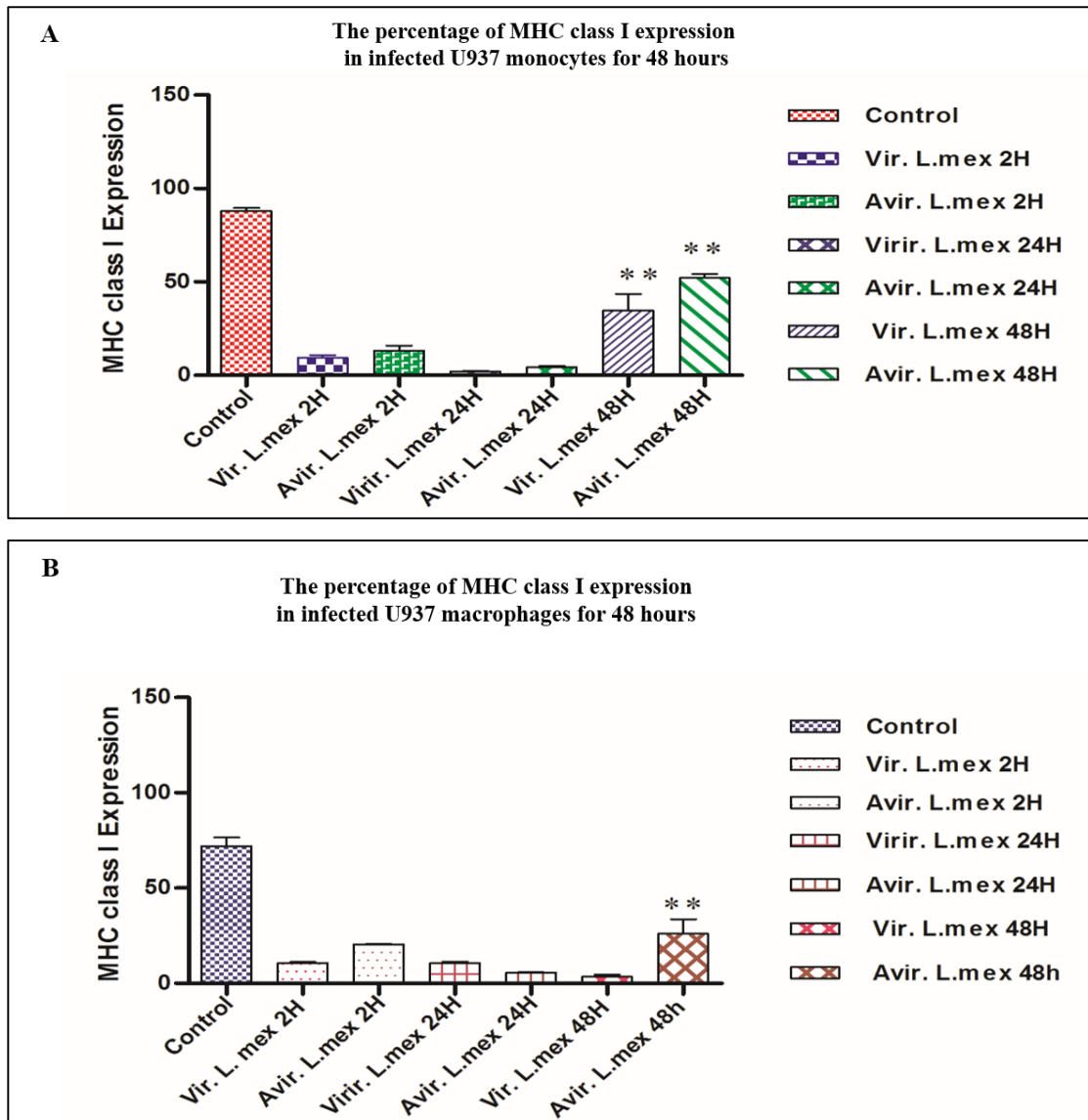


Figure 5.2-10 Spontaneous restoration of MHC class I expression in host cells

U937 monocytes and U937 macrophages were infected with virulent and avirulent *L. mexicana* promastigotes at a ratio of 1:30 (cell: parasites) for up to 48 hours. Target cells were then fixed with 2% w/v paraformaldehyde and stained for MHC class I expression. Data are derived from at least 3 independent experiments for each cell line. ** $P < 0.01$ using the Student's t-test. Data are presented as mean + SEM.

5.3 Discussion

Although the down regulation of MHC class I molecules and neutralization of CTLs activities following infection with intracellular pathogens is well-established following virus infection, there is not much information about the effect of *Leishmania* infection on MHC class I expression (Rappocciolo, *et. al.*, 2002; Yossef, *et. al.*, 2012). Available data are provide various and contradictory findings, which may reflect the complexity and sensitivity of type of studies.

Virus have developed different mechanisms to avoid the antigen presentation by MHC class I and class II. These mechanisms vary according to the virus type. For example infection of human fibroblast with varicella-zoster virus (VZV) has caused significant down regulation of the surface major histocompatibility complex (MHC) class II molecules which can be reversed by pre-treatment with IFN-gamma (Abendroth, *et. al.* 2000)

The down regulation of MHC class I might be due to the virus products. For example, HIV-1 Nef and Tat products have been associated with down regulation of MHC class I Ag presentation. Nef is a protein that is unnecessary for HIV-1 replication, (Kestier, *et. al.*, 1991), but Nef increases the pathogenicity of HIV in several ways, including down-modulation of cell surface MHC class I molecules (Schwartz, *et. al.*, 1996; Le Gall, *et. al.*, 1998).

The experiments described in this chapter were thus designed to investigate and compare the effects of virulent and avirulent *L. mexicana* promastigotes, which reflect the parasite's infectivity at different growth stages on down regulation of MHC class I in infected target cells. Since MHC class I molecules can be found on the plasma surface of most nucleated cells (Peter, 2011) the human monocyte cell line U937 monocyte and MonoMac-6 monocytes and U937 macrophages were used as a model in this study to investigate the quantitative expression of MHC class I molecules following infection with *L. mexicana* promastigotes, using flow cytometry analysis.

To the best of our knowledge, this is the first comprehensive study to investigate the MHC class I expression using different human cell lines infected with different growth stages of *L. mexicana* promastigotes, therefore, there was little information with which to compare the data obtained from this study.

5) MHC class I expression following *L. mexicana* infection

The experiments in this chapter were designed to evaluate the effect of parasite infection on MHC class expression in whole cell population after 24 hours of infection, and the expression data were expressed as a percentage of positive cells and MFI in separate graphs. Interestingly, apart from the infection of U937 cell line with mid log phase virulent and avirulent *L. mexicana*, the MFI analysis gave similar pattern of results as that of the total percentage of MHC class I expression. The results have shed some light on the importance of the passage number and status of the promastigote growth phase, which if controlled may minimize contradictions in the results.

Although both cell lines used in this study were of a monocytic lineage, results have shown different expression patterns of MHC in response to parasite infection, which may reflect differences in response to foreign antigens. Similar variations between different cell lines in response to the same antigen were also reported by Rappocciolo, *et. al.*, (2002), where NBL-6 and EEL cell lines infected with EHV1 differentially down regulated MHC class I on their surface. Unexpectedly, results of the present study show the ability of early log *L. mexicana* promastigotes to down regulate MHC class I expression on the surface of infected U937 monocytes and MonoMac-6 cell lines. These results contradict previous findings, which reported the infectivity of *Leishmania* promastigotes only observed at their late growth phase (Wozencraft, and Blackwell 1987). In a study by Moreira, *et. al.*, (2012), the effect of purified and non-purified (procyclic and metacyclic promastigotes isolated using a Ficoll density purification assay) *L. infantum* promastigotes on the expression of MHC class II molecules and the co-stimulatory antigen CD40 was compared. Their results showed that infection of target cells with non-purified promastigotes at passages P4, P21, and P31 induced different levels of down regulation of these markers. However, these differences were not seen when purified promastigotes were used.

In the current study, different parasite growth stages have been identified *in vitro* for both virulent and avirulent parasites, based on parasite number per ml according to the protocol by Mottram, 2008 (personal communication), as illustrated in Table 5.2.1.B. Further investigation using the published method by Moreira, *et al.*, (2012), was carried out to confirm the enrichment of metacyclic promastigotes when the parasite number reached $\leq 2 \times 10^7$ or above compared to its expression when the promastigote density was less. The results showed a high expression of the *L. mexicana* *Sherp* gene, which has been reported to be up regulated in metacyclic promastigotes (Knuepfer, *et. al.*,

5) MHC class I expression following *L. mexicana* infection

2001). Furthermore, *L. mexicana* histone H4 expression, which has been characterized as a procyclic promastigote gene in *L. infantum* (Soto, *et. al.*, 1997) was highly expressed when promastigote density was less than 2×10^7 , compared to its expression when the parasite number was higher.

The results (Figure 5.2.5) also demonstrated that the expression of *Sherp* gene at the metacyclic stage was greater in virulent parasites compared to avirulent parasites. This may be another reason explaining previous reports on the inability of avirulent parasite to induce the infection (due to failure to reach the metacyclic stage). The analysis of virulency associated genes determined by qPCR confirmed expression up regulation of GP63 and LPG2 genes. These play an important role in parasite attachment to the host cells as well as inhibition of the innate immune response by interfering with the complement system pathway (Isnard, *et. al.*, 2012) at the late log phase compared to the early phase of parasite growth. The expression of CPC, CPC2 and CPC2.8 genes has followed the same pattern. The above genes have an important role in parasite survival inside the host cells. Results also showed that an infection with early log phase promastigotes failed to transform into the amastigote stage after 24 hours infection compared to infection with parasites at late log phase (Figure 5.2.5. A).

This study has illustrated that the stationary phase (defined above as the population peak) can be observed when the parasite was growing in the absence of oxygen, and also the virulence associated genes were highly expressed when parasites were grown anaerobically (Appendix 15). More investigation on the effect of different conditions, such as presence or absence of oxygen, in different types of medium on the associated gene expression profile and parasite infectivity is required.

This study has demonstrated that the down regulation of MHC class I in monocytes and macrophages infected with *L. mexicana* promastigotes at the late phase was infection dose-dependent. Hence, the infection of target cells with *L. mexicana* promastigotes at a ratio of 1:10 (cell: parasites) has no effect on MHC class I expression, compared to infection with ratios of 1:20 and 1:30 (cell: parasite), when the MHC class I expression was gradually decreased using both virulent and avirulent parasites.

Since the complete down regulation of MHC class I in infected cells was achieved at the ratio of infection of 1:30 (cell: parasites), this ratio was used for further analyses. It is important to mention that cell viability was checked, after the complete down regulation of MHC class I expression (Figure 5.2.7), using trypan blue and the MTT assay. The results showed that the cell viability was 98% (data not shown). This was

5) MHC class I expression following *L. mexicana* infection

further confirmed by restoration of MHC I expression after 48 hours of infection of U937 macrophages with 1:30 virulent and avirulent *L. mexicana* promastigotes (Figure 5.2.8.C), which may be related to the ability of these cells to produce and secrete some anti-*Leishmania* reagents. This was also in agreement with the previous results (Figure 4.2.7) which illustrated the number of amastigotes of both virulent and avirulent parasites was significantly decreased in culture of infected U937 monocytes. On the other hand, this finding illustrated why the *Leishmania* parasites survive and proliferate in the macrophages compared to the monocytes, which has been reported by Yang, *et. al* (2007) and Rabhi, *et. al.*, (2010).

One possible explanation for the down regulation of MHC class I in infected U937 monocytes and MonoMac-6 cell lines with early log phase *L. mexicana* promastigotes after 24 hours, is parasite clearance, rather than an evasion mechanism. This theory is supported by the ability of the late log stage to transform and produce the amastigote stage after 24 hours infection, compared to the use of the early log phase, which failed to do so (Figure 5.2.5 B). On the other hand, the inability of late log phase parasites to down regulate MHC class I in infected target cells could be explained by the silent entrance of matured promastigotes, which is considered parasite evasion mechanism (Yang, *et. al.*, 1996).

Interestingly, the results of this study showed that treatment with 4µg per ml Fungizone (an anti-*Leishmania* agent) of target cells infected with late log phase of *L. mexicana* promastigotes for 2 or 24 hours, caused the restoration of the MHC class I expression. The ability of target cells to restore MHC class I expression may affect susceptibility to CTL activity, which is an important element for controlling Leishmaniasis. Because *Leishmania* spp are obligatory intracellular microbes, an adaptive immune response, which includes T helper cell activation and cytokine production is required to control the parasite infection. The process of T cell activation requires parasite antigen presentation by infected APCs *via* MHC class I and MHC class II.

Many studies have identified the significant role of adaptive immune responses to contain infectious diseases such as Leishmaniasis, but this role has been shown mostly to be dependent on host genetic background. For example, macrophages derived from resistant mouse strains (C57BL/6 and C3H) were shown to produce a high level of IFN- γ in response to *Leishmania* infection (Wilson, *et. al.*, 2002; Bourreau, *et. al.*, 2003). In contrast, the infection of susceptible Balb/c mice macrophages with *L. major* was usually accompanied by a Th2 cellular response, which is characterised by high levels

5) MHC class I expression following *L. mexicana* infection

of IL-10 and IL-4 (Rogers, *et. al.*, 2002; Gummy, *et. al.*, 2004). Similar findings have been reported in human Leishmaniasis. For example, an early study by Sundar, *et. al.*, (1997) showed that the level of Th1 associated IFN- γ cytokine production was low compared to IL-4 present in blood samples of individuals with VL. However another study by Hailu, *et. al.*, (2005) showed that the plasma levels of IFN- γ and IL-10 were high in VL patients, and that CD4⁺ and CD8⁺ T cells from these patients failed to produce IL-4 or IFN- γ . These findings have highlighted the important role of MHC class I and class II, which present the parasite antigens to CD8 and CD4 T cells.

Studying the modulation of MHC class I expression in U937 macrophages has highlighted differences between the virulent and avirulent *L. mexicana* effect on MHC class I expression. The results of short time infection (2 hours) have demonstrated a significant down regulation of MHC class I expression in U937 macrophages infected with virulent and avirulent parasites. However, the MHC class I down regulation was greater in cells infected with the virulent parasite. Treatment of infected U937 macrophages with Fungizone caused more restoration of MHC class I molecules in U937 macrophages infected with avirulent compared to virulent parasites. The difference of MHC class I spontaneous restoration between virulent and avirulent parasites was more obvious at 48 hours post-infection in U937 macrophages.

While the expression of MHC class I in U937 macrophage infected with avirulent *L. mexicana* fluctuated among the three tested time points, infection with virulent parasites caused a gradual down regulation of MHC I molecules, with less expression at 48 hours post infection (Figure 5.2.9.C). It has been reported that the survival and proliferation of *Leishmania* parasites inside PVs of infected host cells is dependent on many factors, including their tolerance to the acidic conditions, resistance to degradation by lysosome enzymes, and avoidance of the macrophage's antigen-presenting capacity (Russell, *et. al.*, 1992). The results of this study clearly show that there was little difference in levels of down regulation of MHC class I expression in host cells whether infected with virulent or avirulent *L. mexicana* promastigotes after 2 or 24 hours post-infection. In contrast, long term infection of 48 hours induced a significant restoration of MHC class I expression in U937 macrophages infected with avirulent, but not virulent *L. mexicana* promastigotes. These interesting findings may partly explain the failure of avirulent parasites to induce infection *in vivo*. This also shows that target cells infected with virulent but not avirulent parasite cannot be recognised any more by activated CTLs. Although

5) MHC class I expression following *L. mexicana* infection

not much has been reported on MHC class I regulation by *Leishmania* parasite infection, more is known with respect to virus infection. Rehm, *et. al.*, (2009) have reported that infection with VACV (a live virus vaccine against human smallpox (TC1) and monkey pox viruses) has significantly down regulated MHC class II by APCs, and modulated the synthesis of 13 chemokines and cytokines. This virus has also significantly down regulated NO production in infected RAW macrophages and induced apoptosis.

In this study we have reported that the number of infected cells with virulent, but not avirulent *L. mexicana* promastigotes was significantly decreased, suggesting cell death. In addition, results revealed that infection of U937 macrophages with virulent parasites significantly down regulated the NO production, compared to avirulent infection (data not shown). It has also been reported that regulation of virus virulence genes is associated with antigen presentation. For example Rehm *et. al.*, (2010) has reported that A35R gene up regulation has significantly inhibited MHC II expression, with subsequent effects on chemokine and cytokine synthesis. We have previously reported that infection of U937 macrophages or U937 monocytes with virulent *L. mexicana* promastigotes for 2 hours caused a significant expression of pro-inflammatory cytokines, compared to infection with avirulent parasites, which may correlate with MHC class I expression in cells infected with virulent and avirulent parasites (Ali, *et. al.*, 2013).

Collectively these results have addressed the fact that there are different pathways and mechanisms followed by *L. mexicana* promastigotes once inside their target cells, to down regulate MHC class I. In addition, the results highlight the need to know the status of parasites being investigated, such as passage numbers and growth phase as well as the ratio of infection, in order to minimize the contradictory findings.

**Chapter 6/ Chemokine-Cytokine-Toll like
receptor network response following
infection with *L. mexicana***

6. Chemokine-Cytokine-Toll like receptor network responses following infection with *L. mexicana*

6.1 Introduction

Apart from being the ultimate host cells for *Leishmania* spp, macrophages also have an essential role in the protective immune responses against these parasites through the antigen presentation process, secretion of microbicidal products, and production of inflammatory mediators such as cytokines and chemokines. Early events during host cell-parasite interaction are initiated by Toll-like receptors (TLRs), and these can eventually regulate the outcome of infection (Bosque, *et. al.*, 2009). This is also *Leishmania* spp and host-cell type dependent. This study (as indicated by results from Chapter 5) clearly shows the dramatic effect of *Leishmania* parasite infection on the antigen presentation mechanisms of APCs. The data demonstrated a 30-100% down regulation of MHC class I expression occurred following the infection of susceptible human cell lines with *L. mexicana* promastigotes for 2 and 24 hours, respectively. Unexpectedly, there was no difference in MHC class I expression of cells infected with virulent and avirulent *L. mexicana*, apart from following the infection of U937 macrophages for 48 hours. The results have shed some light on the importance and participation of other immune response elements (such as cytokines, chemokines and toll-like receptors) in the control of parasite infection, which links both innate and adaptive immune response.

Among the three mentioned mediators, cytokines have been considered as the key factor in the immune response to *Leishmania* infection, and have been heavily investigated than the other two mediators (Noben-Trauth, *et. al.*, 1996; Nishikomori, *et. al.*, 2001). Cytokines such as IL-1 β and TNF- α and IL-12 can be produced by macrophages which harbour the parasites. IFN- γ is also produced by Th1 cells and NK cells, whereas IL-4 and IL-10 can be produced by Th2 cells (Sacks and Noben, 2002). In addition to their direct contribution to *Leishmania* infection control or disease exacerbation, cytokines can also up regulate the expression of some chemokines, which in turn induce leukocyte recruitment to the site of the infection (Ohmori, *et. al.*, 1993). For example, IL-1 and TNF- α secreted from infected macrophages or neutrophils induce the expression of chemokines in fibroblasts, endothelial and epithelial cells

6) Chemokines-Cytokines-Toll like receptors network

(Moser, *et. al.*, 2004). The cytokines and chemokines can also work collectively to control the *Leishmania* infection. A study by Zaph and Scottit (2003) reported that IL-12 was an important for the induction of XCL-1, CXCL-10 and CCL-2 expression in lymph nodes which required for *L. major* infection control. Chemokines can be divided into two types. Firstly, Th1 related chemokines such as CXCL-9 and CXCL-10, which are induced by IFN- γ and secondly, Th2 related chemokines such as CCL-22 and CCL-6, which are induced by IL-4 and IL-13 (Bonecchi, *et. al.*, 1998).

Chemokines are involved in both innate and adaptive immune responses to *Leishmania* infection. The immune response to *Leishmania* infection starts immediately following parasite delivery by an infected sand fly (or by deliberate injection in the case of experimental models), which involves parasite interaction with DCs and macrophages via their TLRs (Muzio, *et. al.*, 2000). It has been reported that the saliva of the sand fly contains many mediators that trigger several types of immune response, including the attraction of polymorphonuclear leukocytes (PMNs) to the site of infection (Van, *et. al.*, 2002). In humans, as soon as PMNs cells arrive at the site of *L. major* entry, they start to secrete IL-8 (also known as CXCL-8 chemokine), and CXCL-1 in infected mice. These chemokines in turn attract more PMNs to the site of infection (Mülle, *et. al.*, 2001; Laufs, *et. al.*, 2002). *Leishmania* spp can survive inside PMNs for some time while attracting macrophages to the site of infection, which take up the infected PMNs without activation of microbicidal factors (Van, *et. al.*, 2004). This mechanism enables the parasite to enter the macrophages in a silent manner (Ribeiro, *et. al.*, 2004).

Chemokines also have an indirect role in the adaptive immune response to *Leishmania* infection via the activation of DCs. DCs act as APCs and are considered as a bridge between innate and adaptive immune response. They can become activated by chemokine secretion, which causes up-regulation of the expression of some co-stimulatory molecules (CD54, CD40, CD80, CD86) and IL-12 production (Moll, 2002). The capability of activated DCs to transport the parasites from the site of the infection to Langerhans cells in mice infected with *L. major* has been associated with CCR-2 and CCR-7 expression, since CCR-2 deficient mice were susceptible to *L. major* (Sato, *et. al.*, 2000). Another study has reported that disease progression following infection with *L. donovani* was due to the down regulation of CCR-7, which also inhibited DC activation and their migration to lymph tissue (Ato, *et. al.*, 2002).

Full activation of the immune response relies on recognition of the pathogen through a wide range of molecules occupying the surface of APCs. These molecules, called

6) Chemokines-Cytokines-Toll like receptors network

Toll-Like receptors, which can recognize various microbial antigens and link both the innate and adaptive immune response (Gazzinelli and Denkers, 2006; Medzhitov, 2007). Engagement of TLRs with adaptor proteins such as MyD88 and/or TRIF leads to activation and migration of NF- κ B to the cells' nuclei, subsequently enhancing the transcription of inflammatory mediators such as tumour necrosis factor alpha (TNF- α), and IL-12 (Peter, 2009). The important role of TLRs in *Leishmania* parasite infection has been investigated using genetically modified mice (Liese, *et. al.*, 2008; Tuon, *et. al.*, 2008). For example, many studies have revealed that resistant C57BL/6 mice become susceptible to *L. major* infection when MyD88 and TLR-4 genes are knocked out (Debus, *et. al.*, 2003; Kropf, *et. al.*, 2004; Muraille, *et. al.*, 2003). Other studies have demonstrated that knock out of TLR-9 caused reduction in NK cell activation in mice models following infection with *L. major*, or *L. infantum* (Liese, *et. al.*, 2007; Schleiche, *et. al.*, 2007).

Unlike other micro-organisms, information about the properties and structure of *Leishmania* antigens which interact with different TLRs is incomplete and limited. However, the participation of some of these antigens in the immune response to parasite infection is well documented. For example, *L. major* LPG has been described as a parasite ligand that interacts with TLR-2 and activates human NK cells or murine monocytes (De Veer, *et. al.*, 2003). LPG-TLR-2 interaction induces parasite clearance through nitric oxide (NO) production by human PBMCs (Kavoosi, *et. al.*, 2009). Vargas *et. al.*, (2009) have reported that the P8 proteoglyco-lipid complex (P8 PGLC) expressed on *L. pifano* promastigotes can activate macrophages via TLR-4. Another study has found that DC activation can be induced by *L. major* DNA via TLR-9, which induces the production of IFN- γ by CD4 cells (Fakher, *et. al.*, 2009).

Unlike the mouse model, little is known on the effect of *Leishmania* parasite infection on the network of chemokines, cytokines, and toll-like receptors function in humans. Therefore, the modulation of TLR, interleukin and chemokine expression was investigated. After gaining entry into the host body, *Leishmania* parasites will not only be faced by macrophages, but also by other cell types including monocytes, it was decided to include (U937 monocytes, U937 macrophages and MonoMac-6 monocyte) cell lines in this investigation. In this Chapter the effect of pathogenic (virulent) and non-pathogenic (avirulent) *L. mexicana* promastigotes, or their antigens on host cells was evaluated and compared using qPCR. The dose of infection was chosen based on

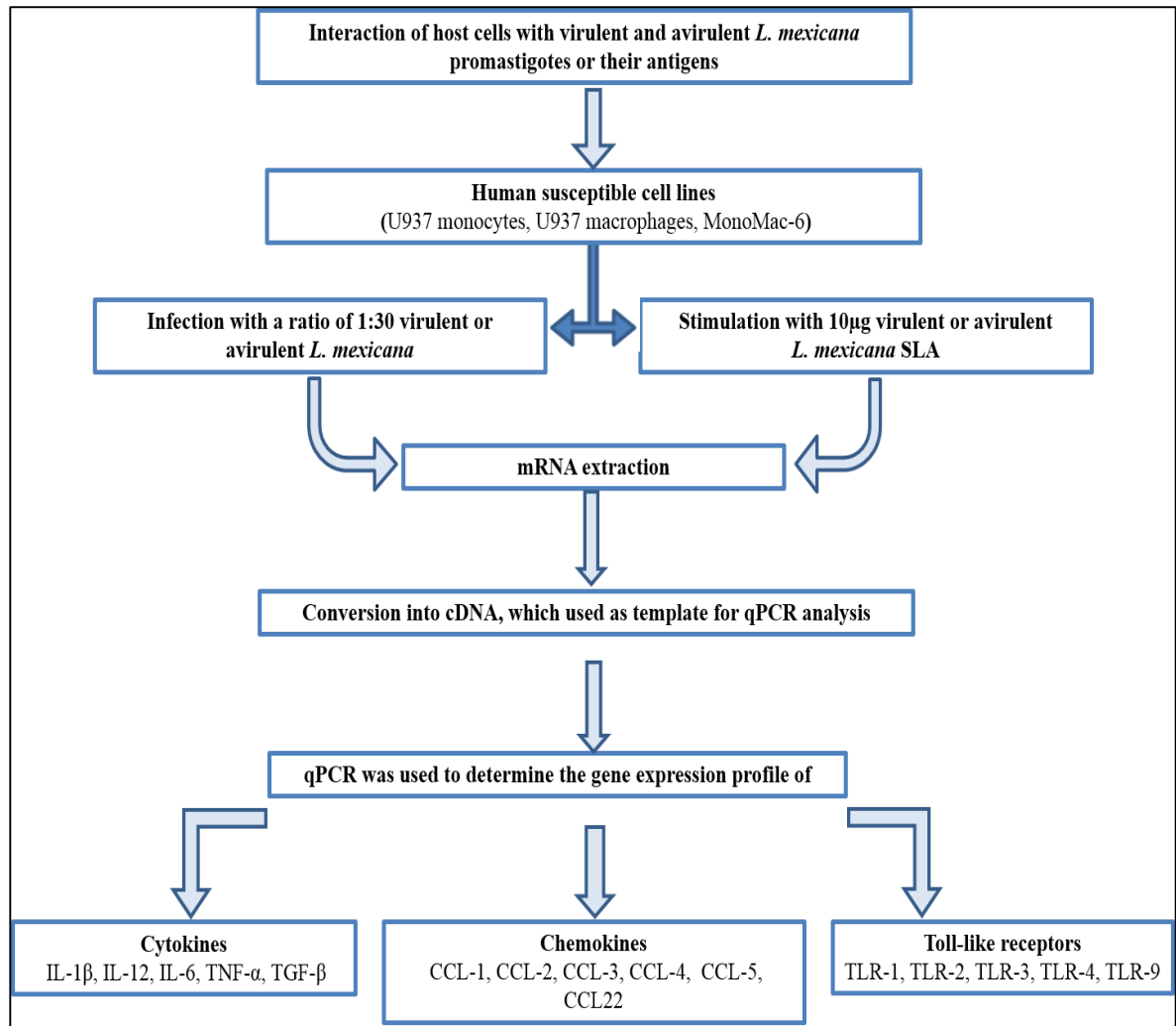
6) *Chemokines-Cytokines-Toll like receptors network*

the findings reported in Chapter 5. A ratio of 1:30 (cell: parasites) was used in this investigation, since this ratio caused the highest down regulation of MHC class I.

6) Chemokines-Cytokines-Toll like receptors network

The work flow

To investigate the effect of virulent and avirulent *L. mexicana* and their antigens on the expression profile of some TLR, interleukin and chemokine genes, experiments were conducted according to the following schedule:



6.2 The effect of *L. mexicana* promastigote infection on TLR, interleukin and chemokine gene expression profiles

These experiments aimed to investigate the changes in the gene expression profile of target cells following infection with *L. mexicana* promastigotes that may help the parasite to escape and evade host immune system defences. The gene expression profile of some Toll-Like receptors (TLRs), cytokines (ILs) and chemokines (CCLs) after infection of three different susceptible human cell lines with *L. mexicana* promastigotes was determined using qPCR. MonoMac-6, U937 monocytes and U937 macrophages were infected with the late log phase of virulent and avirulent *L. mexicana* promastigotes for 24 hours. Total mRNA was extracted from infected and non-infected (control) cells and converted to one strand cDNA, which was used as a DNA template for qPCR analysis.

6.2.1 Baseline gene expression profile of TLRs, ILs and CCLs in non-infected human cell lines

Table 6.2.1 shows the overall expression level of all target genes which varied across the cell lines used. All TLRs were highly expressed in U937 macrophages and U937 monocytes, apart from TLR4, which was not expressed in U937 monocytes. The expression of TLRs was much less in MonoMac-6 cells compared to U937 monocytes and macrophages. Expression of interleukins (ILs) also varied among the different cell lines. IL-1 was similarly expressed in the three cell lines at the same level, in contrast IL-12 cytokines was not detectable in all cells. U937 macrophages only expressed CCL-1, CCL-2, and CCL-5, while U937 monocytes expressed CCL-1, CCL-2, CCL-4 and CCL-5. In spite of their low expression, all chemokines apart from CCL-5 (highly expressed), were detectable in MonoMac-6. CCL-22 was not detected in any of the cell lines.

6) Chemokines-Cytokines-Toll like receptors network

Table 6.2.1 The base line gene expression profile of TLRs, ILs and CCLs in U937 monocytes, U937 macrophages and MonoMac-6 controls (non-infected) cell lines

Targ gene	TLR1	TLR2	TLR4	TLR9	IL1	IL6	IL10	IL12 β	TNF α	TGF β	CCL1	CCL2	CCL3	CCL4	CCL-5	CCL22
U937 Monocytes	++++	++++	-	++++	+	-	+++	-	++	++++	++++	++++	+	++++	++++	-
U927 macrophages	++++	++++	++++	++++	+	+	+++	-	-	++++	++++	++++	-	-	++++	-
MonoMac-6	+	+++	+	+	+	-	+	-	+	++	+	+	+	+	+++	-

Expression level: less than 0.0001(-); between 0.0001 and 0.025 (+); between 0.025 and 0.25 (++);
between 0.25 and 0.75 (+++); more than 0.75 (++++)

6.2.2 The effect of *L. mexicana* promastigote infection on gene expression profile of toll-like receptors (TLRs) in susceptible human cell lines

Table 6.2.2 shows the effect of *L. mexicana* promastigote infection on TLRs gene expression. Although the level of TLRs expression varied according to the host cell lines, general, infection with *L. mexicana* promastigotes caused down regulation of tested TLRs, irrespective of whether the avirulent or virulent parasite was used in the infection.

Typically, infection of U937 monocytes with virulent and avirulent *L. mexicana* promastigotes for 24 hours significantly down regulated the expression of TLR-1, TLR-2 and TLR-9, but not TLR-4, which was significantly up regulated.

The symbol (↓) indicates there was less down regulation induced by the parasite, compared to (↓↓), where more down regulation was observed. In contrast, symbols (↑) and (↑↑) indicated up-regulation or more up-regulation respectively following parasite infection.

The expression of TLR-1, TLR-2 and TLR-9 was significantly down regulated following the infection with virulent compared to avirulent parasite.

The expression of TLRs of non-infected MonoMac-6 was relatively low. Infection of these cells with avirulent *L. mexicana* promastigotes significantly down regulated TLR-2 and TLR-4. In contrast, virulent parasites up regulated TLR-1 and TLR-9.

In summary, the results show that the gene expression profiles of the tested TLRs in this study were different according to the cell line used and the virulence status of the parasite used for infection. The expression of TLR-1 and TLR-9 was down regulated after parasite infection across all tested cell lines, except the infection of MonoMac-6 cells with virulent *L. mexicana* promastigotes, which up-regulated the expression of these genes. TLR-2 gene expression was down regulated following parasite infection across all tested cell lines. TLR-4 expression has a similar pattern to that of TLR-2, except the infection of U937 monocytes with *L. mexicana* promastigotes, which caused an up-regulation of TLR-4 expression. Some of the untransformed qPCR data are shown in Figure 6.2.1.

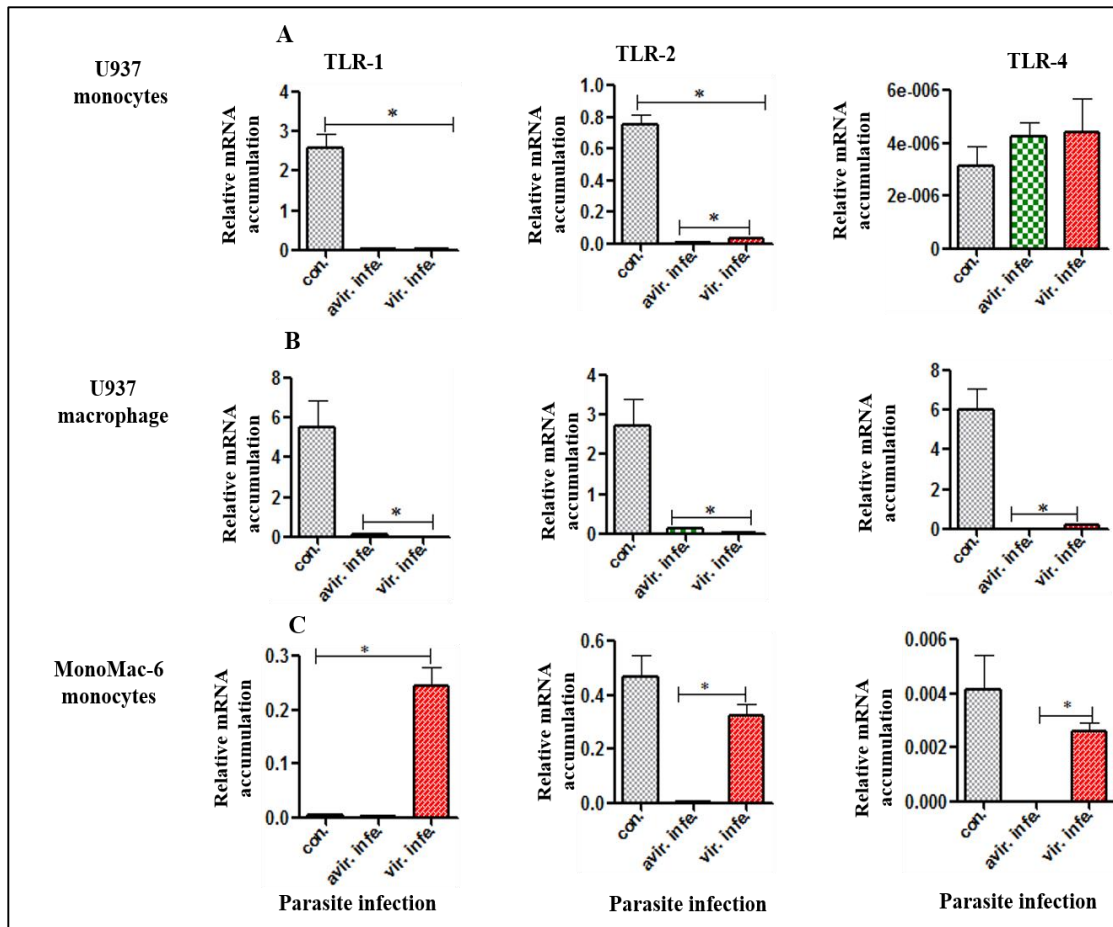
6) Chemokines-Cytokines-Toll like receptors network

Table 6.2.2 The gene expression profile of TLRs after infection of three different cell lines with avirulent and virulent *L. mexicana*

		TLR-1	TLR-2	TLR-4	TLR-9
U937 Monocytes	control	++++	++++	-	++++
	Infected avirulent	↓*	↓*	↑*	↓↓* #
	Infected virulent	↓↓* #	↓↓* #	↑*	↓*
U937 macrophage	control	++++	++++	++++	++++
	Infected avirulent	↓*	↓*	↓↓*#	↓*
	Infected virulent	↓↓*#	↓↓*#	↓*	↓↓* #
MonoMac-6	control	+	+++	+	+
	Infected avirulent	↓	↓↓*#	↓↓*#	↓*
	Infected virulent	↑↑* #	↓	↓*	↑↑*#

Expression level: less than 0.0001(-); between 0.0001 and 0.025 (+); between 0.025 and 0.25 (++); between 0.25 and 0.75 (+++); more than 0.75 (++++). The Mann-Whitney U-test was used for statistical analysis and a P value of < 0.05 was taken as significant. ↓ one arrow indicates the presence of inhibition. ↑ indicated the presence of up-regulation. ↓↓ indicates the presence of more inhibition compared with ↓; ↑↑ indicates the presence of more up-regulation compared with ↑. * indicates the presence of a significant difference between infection and control cells. # indicates the presence of a significant difference between virulent and avirulent *L. mexicana* promastigotes. Shaded cells indicate a difference in regulation between virulent and avirulent parasites using the same cell line.

6) Chemokines-Cytokines-Toll like receptors network



Finger 6.2.1 The effect of *L. mexicana* infection on gene expression profile of Toll-like receptors (TLRs)

Target cells were infected with virulent or avirulent *L. mexicana* promastigotes for 24 hours. RNA was extracted from control and infected cells. Extracted RNA was converted to cDNA, which was used as a template for qPCR analysis. Data are the averages of least 3 independent experiments, *P<0.05 was calculated using the Mann-Whitney test. Data are presented as mean + SEM.

6.2.3 The effect of *L. mexicana* promastigote infection on gene expression profile of cytokines (ILs) in susceptible human cell lines

Table 6.2.3 shows the effect of *L. mexicana* promastigote infection on the gene expression profile of cytokines (ILs) measured by qPCR these also varied depending on the cell line tested. Like TLRs, the gene expression profile of ILs changed after infection of target cells with *L. mexicana* promastigotes. Results clearly show that the gene expression profile of IL-1 β , IL-6 and IL-12B was significantly up regulated in U937 monocytes infected with the avirulent parasite, while IL-10 expression was down regulated. The results in Table 6.2.3 also show a similar down regulation pattern of gene expression profile of TNF- α following infection of U937 monocytes and MonoMac-6 cells with avirulent and virulent parasites. In contrast, this cytokine was up regulated following the infection of U937 macrophages, and virulent *L. mexicana* induced more expression compared to avirulent parasites.

The TGF- β expression profile in infected U937 macrophages was the same as the TNF- α expression profile, however, TGF- β expression was down regulated in infected U937 monocytes and MonoMac-6 cell lines (except for infection with virulent parasites). Although the base-line level of ILs gene expression was not identical for all tested cytokines in non-infected U937 macrophages, infection of U937 macrophages with *Leishmania* parasite caused a significant up-regulation of IL-1 β , IL-6 (except where there was infection with virulent parasites), IL-12B, TNF- α and TGF- β expression. In contrast, infection with *L. mexicana* promastigotes caused a remarkable down regulation of IL-10 expression. Like the infected U937 monocytes, infection of macrophages with avirulent *L. mexicana* promastigotes also up-regulated IL-1 β , IL-6 more than with virulent parasites. However, infection with virulent *L. mexicana* caused a notable increase in IL-12B, TNF- α and TGF- β expression in comparison with infection with avirulent parasites.

The expression of interleukins in infected MonoMac-6 was also altered. The results show that the gene expression profile of TNF- α and IL-10 cytokines after *Leishmania* parasite infection significantly decreased compared to non-infected MonoMac-6. In contrast, the level of IL-6 and IL-12B expression was considerably up-regulated. IL-1 β cytokines was significantly up-regulated by infection with avirulent parasites, and down regulated after infection with virulent *L. mexicana* promastigotes.

6) Chemokines-Cytokines-Toll like receptors network

Finally, *L. mexicana* promastigote infection has a major impact on interleukins gene expression profiles in the cell lines used. IL-12B expression was up regulated, in contrast to IL-10 which was down regulated following *L. mexicana* infection. Moreover, the levels of IL-1 β and IL-6 were up-regulated across the used cell lines compared to non-infected cells, whereas the levels of TNF- α and TGF- β cytokines was down regulated in infected monocyte cell lines (U937 and MonoMac-6, except where there was infection with the virulent parasite) and up-regulated in infected U937 macrophages. Some of the untransformed qPCR data are shown in Figure 6.2.2.

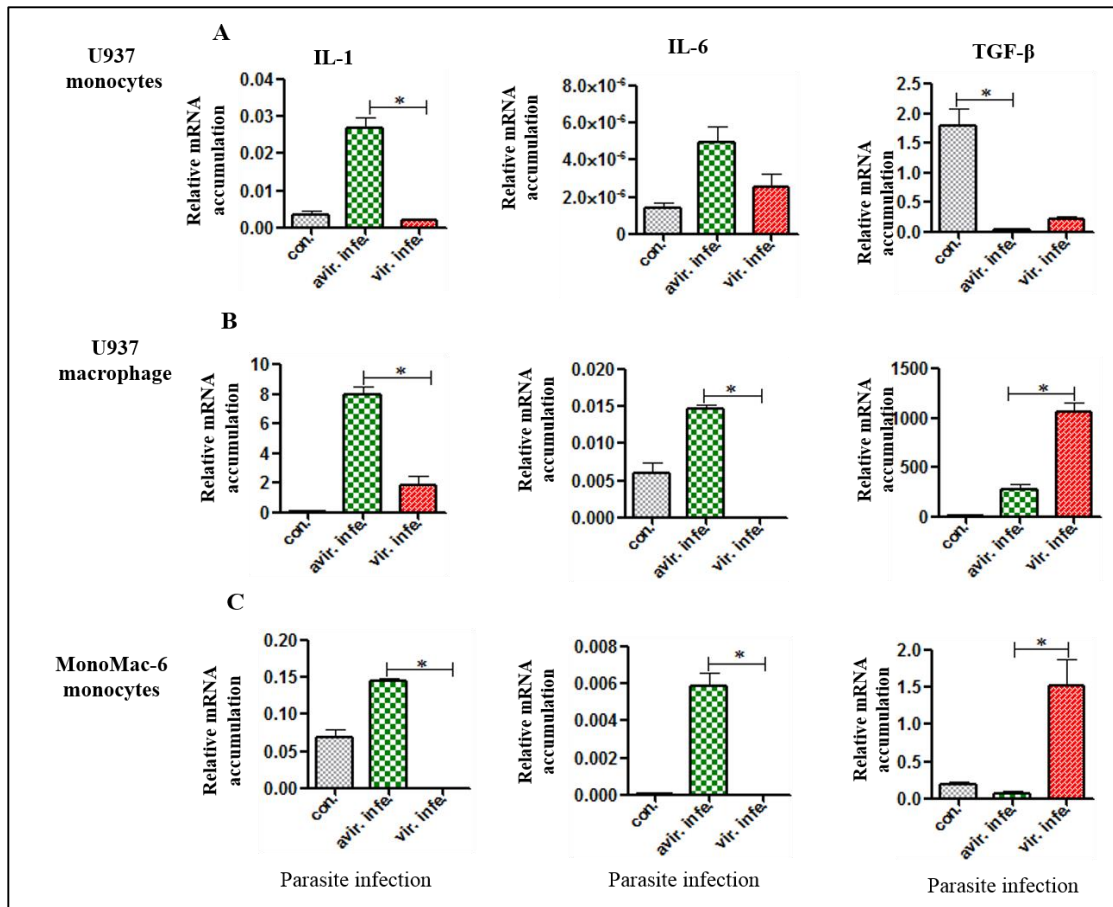
6) Chemokines-Cytokines-Toll like receptors network

Table 6.2.3 The gene expression profile of ILs after infection of three different cell lines with avirulent and virulent *L. mexicana*

		IL-1β	IL-6	IL-10	IL-12B	TNF-α	TGF-β
U937 monocytes	control	+	-	+++	-	++	++++
	Infected avirulent	↑↑* #	↑↑	↓*	↑↑* #	↓*	↓↓* #
	Infected virulent	↓	↑	↓↓* #	↑*	↓↓* #	↓*
U937 macrophage	control	+	+	+++	-	-	++++
	Infected avirulent	↑↑* #	↑↑* #	↓↓* #	↑*	↑*	↑*
	Infected virulent	↑*	↓*	↓*	↑↑* #	↑↑* #	↑↑* #
MonoMac-6	control	++	-	+	-	+	++
	Infected avirulent	↑↑* #	↑↑* #	↓↓* #	↑*	↓*	↓
	Infected virulent	↓*	↑*	↓*	↑↑* #	↓↓* #	↑↑* #

Expression level: less than 0.0001(-); between 0.0001 and 0.025 (+); between 0.025 and 0.25 (++); between 0.25 and 0.75 (+++); more than 0.75 (++++). The Mann-Whitney U-test was used for statistical analysis and a value of $P < 0.05$ was taken as significant. ↓ one arrow indicates the presence of inhibition. ↑ indicates the presence of an up-regulation. ↓↓ indicates the presence of more inhibition compared with ↓; ↑↑ indicates the presence of more up-regulation compared with ↑. * indicates the presence of a significant difference between infection and control cells. # indicates the presence of a significant difference between virulent and avirulent *L. mexicana* promastigotes. Shaded cells indicate a difference in regulation between virulent and avirulent parasites using the same cell line.

6) Chemokines-Cytokines-Toll like receptors network



Finger 6.2.2 The effect of *L. mexicana* infection on gene expression profile of Cytokines (ILs)

Target cells were infected with virulent or avirulent *L. mexicana* promastigotes for 24 hours. RNA was extracted from control and infected cells. Extracted RNA was converted to cDNA, which was used as a template for qPCR analysis. Data are the averages of least 3 independent experiments, *P<0.05 was calculated using the Mann-Whitney test. Data are presented as mean + SEM.

6.2.4 The effect of *L. mexicana* promastigote infection on gene expression profile of chemokines (CCLs) in susceptible human cell lines

Infection with the *Leishmania* parasite significantly affected the gene expression profile of CCL-1, CCL-2, CCL-3, CCL-4, CCL-5 and CCL-22 chemokines, by either down or up-regulated their expression in all cell lines used in this study (Table 6.2.4).

Interestingly, *L. mexicana* infection of U937 monocytes caused a remarkable down regulation of all chemokines (CCL-1, CCL-2, CCL-4 and CCL-5) that were highly expressed in control (non-infected) cells. However, chemokines which were initially not expressed (CCL-22), or expressed at a low level (CCL-3) in non-infected cells were significantly up-regulated. Moreover, results in Table 6.2.4 show that the infection of U937 monocytes with virulent *L. mexicana* significantly down regulated CCL-1, CCL-2 and CCL-4 expression when compared to avirulent parasites.

The level of CCL (except CCL-5) expression was significantly up regulated after infection of U937 macrophages with *Leishmania* parasites (Table 6.2.4). The expression of CCL-1, CCL-2, and CCL-4 chemokines was significantly higher after infection of U937 macrophages with avirulent *L. mexicana* promastigotes when compared to infection with virulent parasites. On the other hand, infection with virulent parasites caused a notable increase in the level of expression of CCL-3 and CCL-22. Avirulent parasite infection induced more down regulation of CCL-5 in comparison to infection by virulent parasites.

Unlike U937 monocytes, the infection of MonoMac-6 with *L. mexicana* promastigotes caused up-regulation of CCL-1, CCL-2, and CCL-4, and down regulated the expression of CCL-22 chemokines. Interestingly, infection of MonoMac-6 with virulent *L. mexicana* promastigotes caused a significant increase of CCL-1, CCL-2, and CCL-4 expression compared with avirulent parasites. Some of the untransformed qPCR data are shown in Figure 6.2.3.

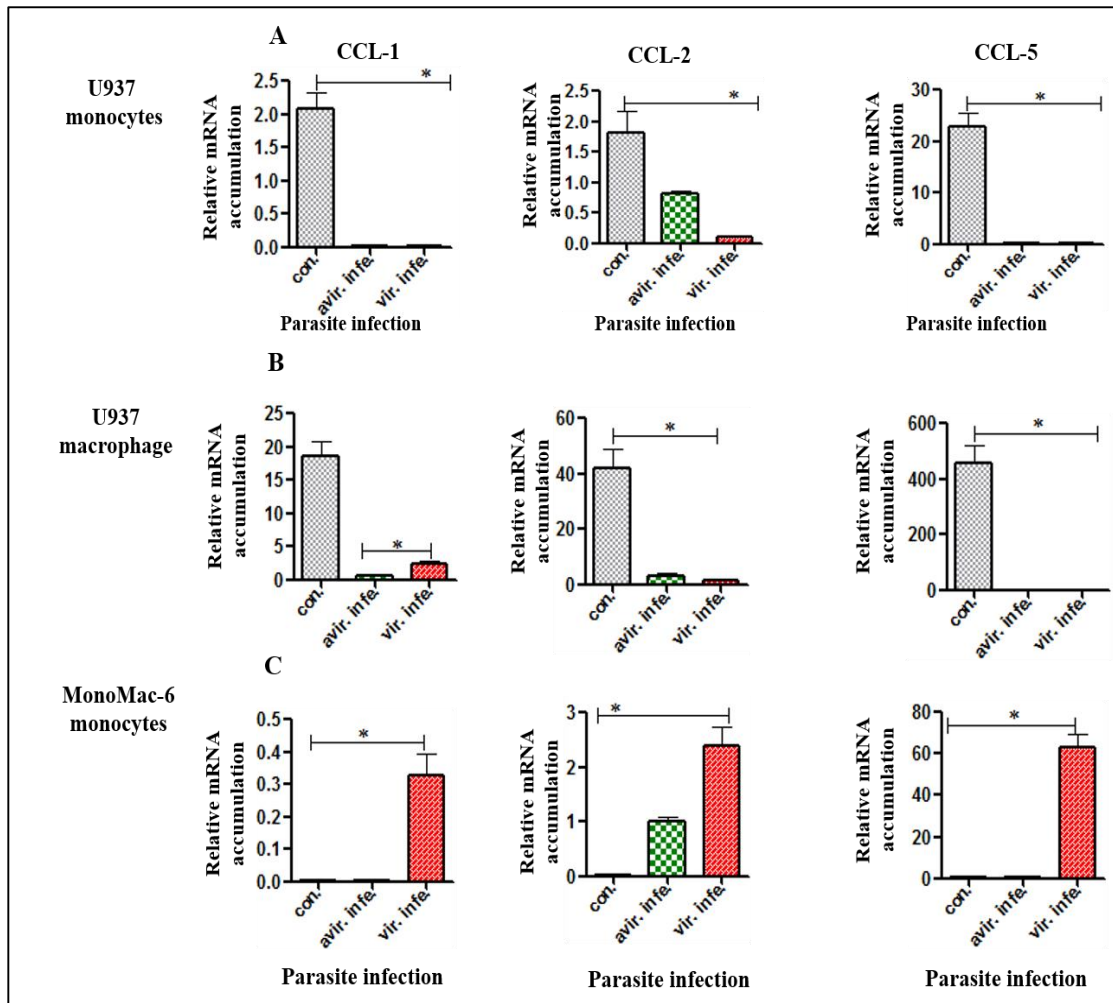
6) Chemokines-Cytokines-Toll like receptors network

Table 6.2.4 The gene expression profile of CCLs after infection of three different cell lines with avirulent and virulent *L. mexicana*

		CCL-1	CCL-2	CCL-3	CCL-4	CCL-5	CCL-22
U937 monocytes	control	++++	++++	+	++++	++++	-
	Infected Avirulent	↓*	↓*	↑↑*	↓*	↓↓*#	↑↑*#
	Infected virulent	↓↓*#	↓↓*#	+	↓↓*#	↓*	↑*
U937 macrophage	control	++++	++++	-	-	++++	-
	Infected Avirulent	↑↑*#	↑↑*#	↑*	↑↑*#	↓↓*#	↑*
	Infected virulent	↑*	↑*	↑↑*#	↑*	↓*	↑↑*#
MonoMac-6	control	+	+	+	+	+++	-
	Infected Avirulent	↑*	↑*	↑*	↑*	↓*	↓↓*#
	Infected virulent	↑↑*#	↑↑*#	↑*	↑↑*#	↑*	↓*

Expression level: less than 0.0001(-); between 0.0001 and 0.025 (+); between 0.025 and 0.25 (++); between 0.25 and 0.75 (+++); more than 0.75 (++++). The Mann-Whitney U-test was used for statistical analysis and a value of $P < 0.05$ was taken as significant. ↓ one arrow indicates the presence of an inhibition. ↑ indicates the presence of an up-regulation. ↓↓ indicates the presence of more inhibition compared with ↓; ↑↑ indicates the presence of more up-regulation compared with ↑. * indicates the presence of a significant difference between infection and control cells. # indicates the presence of a significant difference between virulent and avirulent *L. mexicana* promastigotes. Shaded cells indicate a difference in regulation between virulent and avirulent parasites using the same cell line.

6) Chemokines-Cytokines-Toll like receptors network



Finger 6.2.3 The effect of *L. mexicana* infection on gene expression profile of Chemokines (CCLs)

Target cells were infected with virulent or avirulent *L. mexicana* promastigotes for 24 hours. RNA was extracted from control and infected cells. Extracted RNA was converted to cDNA, which was used as a template for qPCR analysis. Data are the averages of least 3 independent experiments, * $P < 0.05$ was calculated using the Mann-Whitney test. Data are presented as mean + SEM.

6.3 The effect of *L. mexicana* promastigote SLA on the gene expression profile of TLRs, ILs and CCLs

To investigate and compare the immunogenicity of proteins extracted from virulent and avirulent *L. mexicana* promastigotes, human susceptible cell lines were stimulated with avirulent and/or virulent *L. mexicana* SLA. The level of gene expression profile of chemokines, cytokines and Toll-like receptors after stimulation of three different susceptible human cell lines with SLAs was determined using qPCR technique. Human MonoMac-6 cell line, U937 monocytes and U937 macrophages were cultured in 24 well plates at a concentration of 2×10^5 per well, and stimulated with 10 µg per ml of avirulent and/or virulent SLA for 24 hours. Total mRNA was extracted, from each target cell, and converted into one strand cDNA as previously described, to be used as a DNA template for qPCR.

6.3.1 The effect of stimulation with *L. mexicana* promastigote SLA on gene expression profile of toll-like receptors (TLRs) in susceptible human cell lines.

Table 6.3.1 shows the effect of stimulation with *L. mexicana* promastigote SLA on the expression profile of TLRs measured by qPCR. Stimulation of U937 monocytes with virulent *L. mexicana* SLA for 24 hours significantly down regulated the expression of TLR1, TLR2, TLR-4 and TLR-9.

Control (non-infected) U937 macrophages were highly expressed all tested TLRs, and these were significantly down regulated after stimulation with *L. mexicana* SAL, (Table 6.3.1). The results clearly show that virulent SLA induced more down regulation of TLR-1 and TLR-9 compared to stimulation with avirulent *L. mexicana* promastigote SLA, which induced more down regulation of TLR-2.

The level of TLRs gene expression profile of the control MonoMac-6 was relatively low compared with that of U937 macrophages. A 24-hour stimulation with SLA significantly down regulated the expression of TLR-2, TLR-4 and TLR-9. However, TLR-1 was significantly up regulated following stimulation with avirulent SLA.

In summary, the results show that the gene expression profiles of tested TLRs in this study were different according to the cell line and the status of the SLA (virulent or avirulent). The expression of TLR-2 and TLR-9 was down regulated after SLA stimulation in the three cell lines. TLR-1 gene expression profile was also down regulated following SLA stimulation across all tested cell lines, except in the case of stimulation of MonoMac-6 cells with virulent SLA. TLR-4 gene expression was also down regulated as a result of stimulation with *L. mexicana* SLA (except in U937 monocytes stimulated with avirulent SLA). Some of the untransformed qPCR data are shown in Figure 6.3.1.

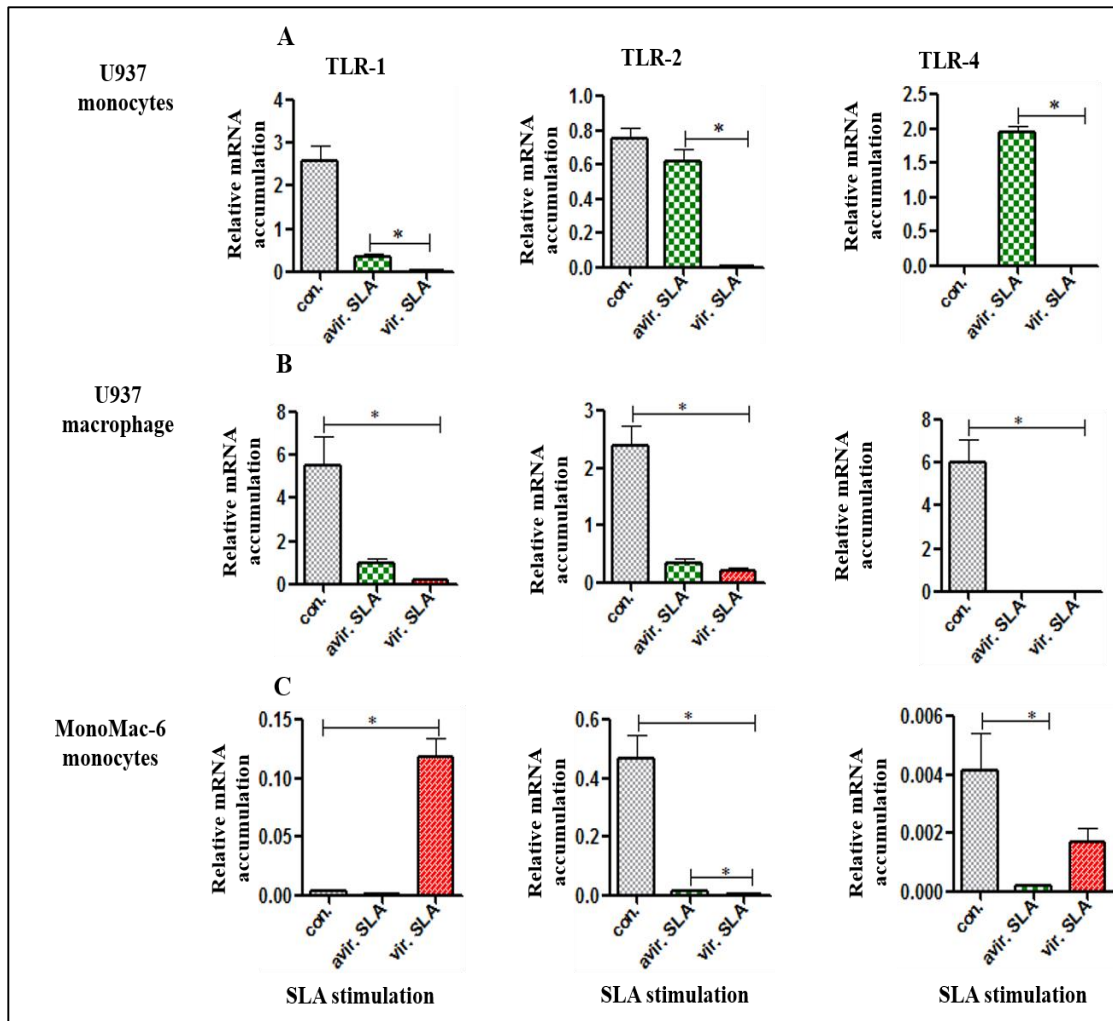
6) Chemokines-Cytokines-Toll like receptors network

Table 6.3.1 The gene expression profile of TLRs after stimulation of three different cell lines with avirulent and virulent *L. mexicana* promastigotes SLA

		TLR-1	TLR-2	TLR-4	TLR-9
U937 monocytes	Control	++++	++++	-	++++
	Avirulent SLA	↓*	↓↓* #	↑↑* #	↓*
	Virulent SLA	↓↓* #	↓*	↑	↓↓* #
U937 macrophage	Control	++++	++++	++++	++++
	Avirulent SLA	↓*	↓*	↓*	↓*
	Virulent SLA	↓↓* #	↓↓*	↓↓* #	↓↓* #
MonoMac-6	Control	+	+++	+	+
	Avirulent SLA	↓*	↓*	↓↓*	↓↓* #
	Virulent SLA	↑↑* #	↓↓* #	↓	↓*

Expression level: less than 0.0001(-); between 0.0001 and 0.025 (+); between 0.025 and 0.25 (++); between 0.25 and 0.75 (+++); more than 0.75 (++++). The Mann-Whitney U-test was used for statistical analysis where a value of $P < 0.05$ was taken as significant. ↓ one arrow indicates the presence of an inhibition. ↑ indicates the presence of an up-regulation. ↓↓ indicates the presence of more inhibition compared with ↓; ↑↑ indicates the presence of more up-regulation compared with ↑. * indicates the presence of a significant difference between stimulated and control cells. # indicates the presence of a significant difference between stimulation with virulent and avirulent *L. mexicana* promastigotes SLA. Shaded cells indicate a difference in regulation between virulent and avirulent parasites using the same cell line.

6) Chemokines-Cytokines-Toll like receptors network



Finger 6.3.1 The effect of the SLA stimulation on gene expression profile of Toll-like receptors (TLRs)

Target cells were stimulated with 10µg virulent or avirulent *L. mexicana* SLA for 24 hours. RNA was extracted from control, infected and stimulated cells and converted to cDNA, which was used as a template for qPCR analysis. Data are the averages of least 3 independent experiments, *P<0.05 was calculated using the Mann-Whitney test. Data are presented as mean + SEM.

6.3.2 The effect of stimulation with *L. mexicana* promastigote SLA on gene expression profile of cytokines (ILs) in susceptible human cell lines.

The effect of *L. mexicana* promastigote SLA on the level of gene expression profile of cytokines (ILs) as measured by qPCR also varied depending on the cell lines used, (Table 6.3.2).

Stimulation of U937 monocytes with 10 μ g SLA per ml significantly down regulated the expression of IL-10 and TNF- α gene. In contrast, IL-6 and IL-12B cytokines were considerably up regulated after stimulation with avirulent SLA. IL-1 was significantly up regulated following stimulation of U937 monocytes with virulent SLA. TGF- β expression was significantly up regulated after SLA stimulation, and the level of expression was greater as a result of stimulation with avirulent in comparison to virulent SLA. In spite of variation in the baseline levels of interleukin gene expression in U937 macrophages, stimulation with avirulent *L. mexicana* SLA caused a considerable up-regulation of IL-12B, TNF- α , and TGF- β , compared to the level of the expression induced by virulent parasites.

Like infection with *Leishmania* parasites, stimulation with SLA caused a significant down regulation of IL-10 expression. Moreover, down regulation produced by stimulation with virulent SLA was significantly greater compared to that caused by stimulation with avirulent SLA. Interestingly, IL-6 expression was significantly up regulated following stimulation with avirulent and down regulated with virulent SLA. However, IL-1 expression was up regulated after stimulation with avirulent SLA, and up regulated even more by virulent SLA stimulation. Similarly, stimulation of MonoMac-6 with 20 μ g SLA changed the level of ILs expression.

IL-1 β , IL-10 and TNF- α gene expression were significantly down regulated following SLA stimulation. In contrast, the level of IL-12B expression was significantly up regulated, but on the other hand IL-6 was significantly up and down regulated after stimulation of MonoMac-6 with avirulent and virulent *L. mexicana* SLA, respectively. TGF- β expression was also down and up regulated after stimulation with avirulent and virulent SLA.

In general, treatment of target cells with SLA has a major impact on interleukin gene expression profile across the used cell lines. IL-1 β expression was up regulated in macrophages and down regulated in both monocytes (apart from stimulation of U937

6) *Chemokines-Cytokines-Toll like receptors network*

cells with virulent SLA). IL-6 expression was up regulated after stimulation with avirulent SAL, but was down regulated following stimulation with virulent SLA. IL-10 expression was down regulated after treatment of cells with SLA. IL-12B expression was up regulated in U937 macrophages and MonoMac-6 cells, whereas TNF- α expression was marginally regulated in U937 macrophages, and down regulated in the monocytes (U937 and MonoMac-6). TGF- β expression was also up regulated after SLA stimulation in U937 monocytes and U937 macrophages. Some of the untransformed qPCR data are shown in Figure 6.3.2.

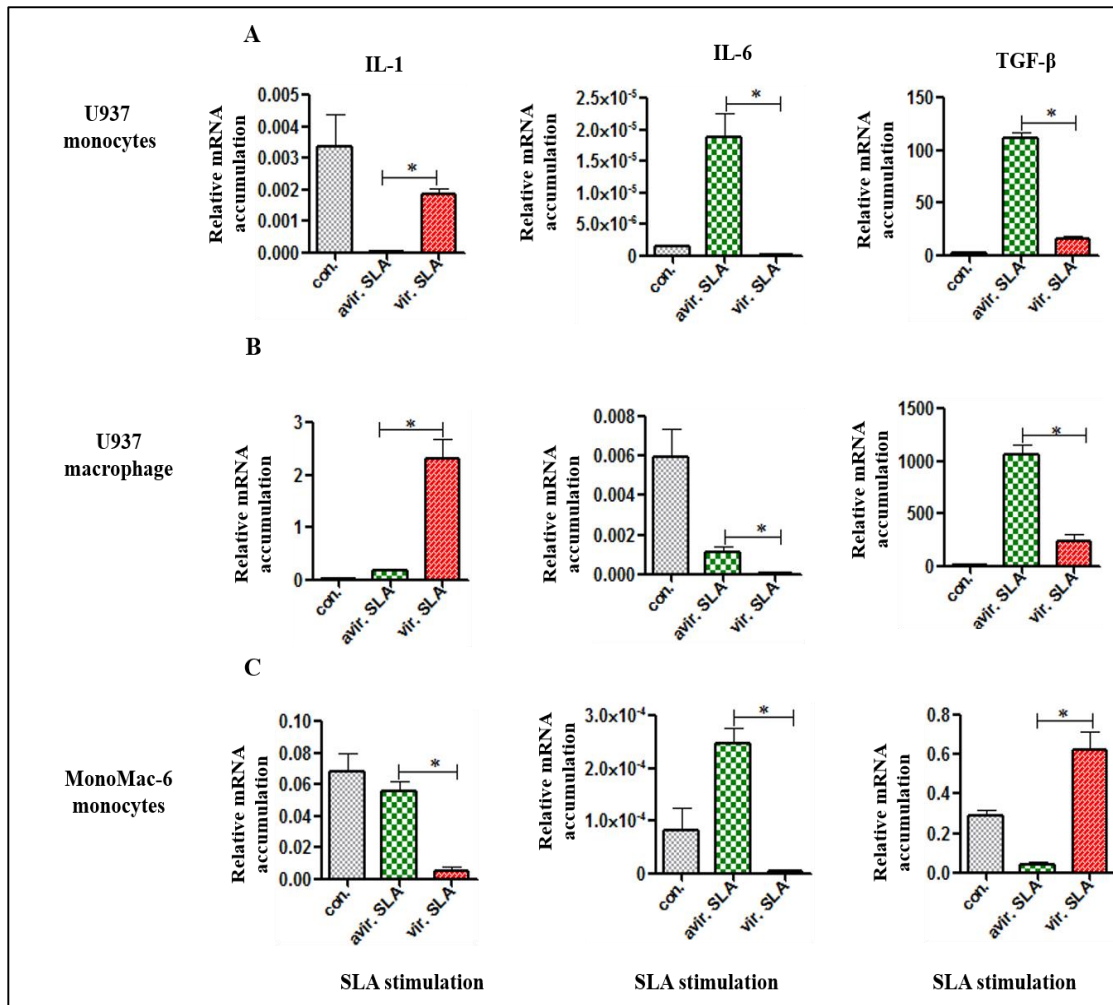
6) Chemokines-Cytokines-Toll like receptors network

Table 6.3.2 The gene expression profile of ILs after stimulation of three different cell lines with avirulent and virulent *L. mexicana* promastigotes SLA

		IL-1β	IL-6	IL-10	IL-12B	TNF-α	TGF-β
U937 monocytes	Control	+	-	+++	-	++	++++
	Avirulent SLA	↓*	↑*	↓*	↑*	↓↓* #	↑↑* #
	Virulent SLA	↑↑* #	↓↓* #	↓↓* #	↓↓* #	↓*	↑*
U937 macrophage	Control	+	+	+++	-	-	++++
	Avirulent SLA	↑*	↓*	↓*	↑↑* #	↑↑* #	↑↑* #
	Virulent SLA	↑↑* #	↓↓* #	↓↓* #	↑*	↑*	↑*
MonoMac-6	Control	+	-	+	-	+	++
	Avirulent SLA	↓*	↑*	↓*	↑↑* #	↓*	↓*
	Virulent SLA	↓↓* #	↓↓* #	↓↓* #	↑*	↓↓* #	↑*

Expression level: less than 0.0001(-); between 0.0001 and 0.025 (+); between 0.025 and 0.25 (++); between 0.25 and 0.75 (+++); more than 0.75 (++++). The Mann-Whitney U-test was used for statistical analysis. A value of $P < 0.05$ was taken as significant. ↓ one arrow indicates the presence of an inhibition. ↑ indicates the presence of an up-regulation. ↓↓ indicates the presence of more inhibition compared with ↓; ↑↑ indicates the presence of more up-regulation compared with ↑. * indicates the presence of a significant difference between stimulated and control cells. # indicates the presence of a significant difference between stimulation with virulent and avirulent *L. mexicana* promastigotes SLA. Shaded cells indicate a difference in regulation between virulent and avirulent parasites using the same cell line.

6) Chemokines-Cytokines-Toll like receptors network



Finger 6.3.2 The effect of the SLA stimulation on gene expression profile of Cytokines (ILs)

Target cells were stimulated with 10μg virulent or avirulent *L. mexicana* SLA, for 24 hours. RNA was extracted from control, infected and stimulated cells and converted to cDNA which was used as a template for qPCR analysis. Data are the averages of least 3 independent experiments, *P<0.05 was calculated using the Mann-Whitney test. Data are presented as mean + SEM.

6.3.3 The effect of stimulation with *L. mexicana* promastigote SLA on gene expression profile of chemokines (CCLs) in susceptible human cell lines.

Stimulation of target cells with *Leishmania* parasite SLA has a wide range of significant effects on the gene expression profile of CCL-1, CCL-2, CCL-3, CCL-4, CCL-5 and CCL-22 chemokines depending on the virulence of parasites used for SLA preparation, (Table 6.3.3).

Notable effects were observed on the level of chemokine gene expression after stimulation of U937 monocytes cell line. *L. mexicana* SLA significantly down regulated CCL-1, CCL-3 and CCL-4 chemokines. Moreover, the results show that stimulation of U937 monocytes cells with virulent SLA caused a significant down regulation of CCL-1, CCL-4, CCL-5 gene expression compared to avirulent SLA. Table 6.3.3 also shows that the level of chemokine gene expression was up regulated after stimulation of U937 macrophages with *Leishmania* parasite SLA.

The level of expression of CCL-1 and CCL-2 chemokines was significantly down regulated after stimulation of U937 macrophages with avirulent SLA compared to avirulent SLA stimulation. CCL-5 expression was significantly down regulated as a result of stimulation with virulent. In contrast, both avirulent and virulent SLA stimulation induced up regulation of CCL-22. Stimulation of MonoMac-6 with virulent *L. mexicana* SLA caused significant up regulation of all tested chemokines. whilst the expression of CCL-1 and CCL-22 chemokines were down and up regulated after treatment with avirulent and virulent SLA respectively.

To summarise, the gene expression of tested chemokine was altered following the stimulation of target cells with *L. mexicana* SLA (Table 6.3.3). A similar pattern was observed on the level of CCL-3 and CCL-4 chemokines (except U937 when stimulated with avirulent SLA) across tested cell lines. The expression of CCL-1 and CCL-2 had a similar pattern in immature U937 monocytes and U937 macrophages. CCL-22 expression was up regulated after SLA stimulation (except MonoMac-6 cells stimulated with avirulent SLA). Some of the untransformed qPCR data are shown in Figure 6.3.3.

6) Chemokines-Cytokines-Toll like receptors network

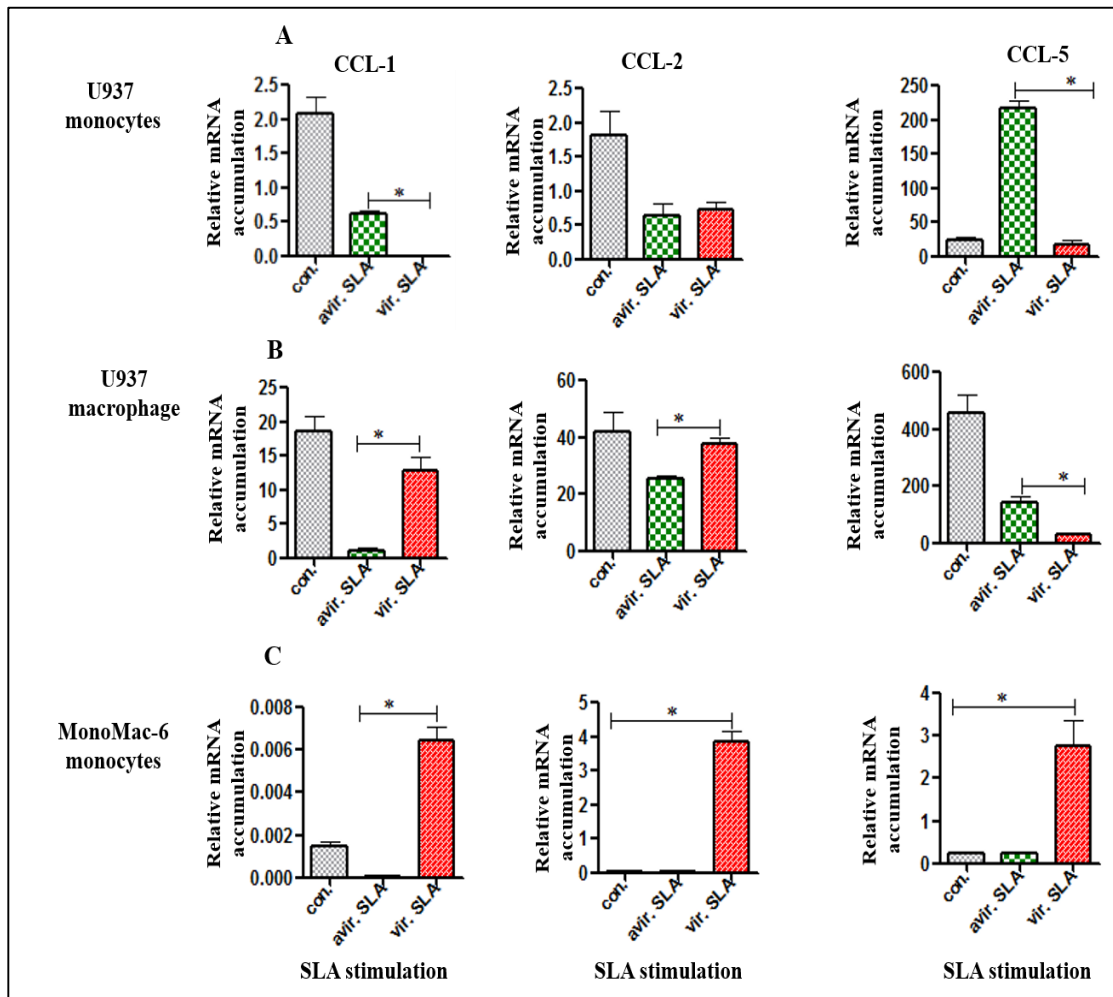
Table 6.3.3 The gene expression profile of CCLs after stimulation of three different cell lines with avirulent and virulent *L. mexicana* promastigotes SLA

		CCL-1	CCL-2	CCL-3	CCL-4	CCL-5	CCL-22
U937 monocytes	Control	++++	++++	+	++++	++++	-
	Avirulent SLA	↓*	↓	↓↓*#	↓*	↑↑*#	↑↑*#
	Virulent SLA	↓↓*#	↓	↓*	↓↓*#	↓	↑*
U937 macrophage	Control	++++	++++	-	-	++++	-
	Avirulent SLA	↓↓*#	↓↓*#	↑*	↑*	↓*	↑*
	Virulent SLA	↓	↓	↑↑*#	↑↑*#	↓↓*#	↑*
MonoMac-6	Control	+	+	+	+	+++	-
	Avirulent SLA	↓*	ND	↑*	↑*	ND	↓*
	Virulent SLA	↑↑*#	↑*#	↑↑*#	↑↑*#	↑*#	↑*

Expression level: less than 0.0001(-); between 0.0001 and 0.025 (+); between 0.025 and 0.25 (++); between 0.25 and 0.75 (+++); more than 0.75 (++++). The Mann-Whitney U-test was used for statistical analysis. A value of P < 0.05 was taken as significant. ↓ one arrow indicates the presence of inhibition. ↑ indicates the presence of an up-regulation. ↓↓ indicates the presence of more inhibition compared with ↓. ND no significant different.

↑↑ indicates the presence of more up-regulation compared with ↑. * indicates the presence of a significant difference between stimulated and control cells. # indicates the presence of a significant difference between stimulation with virulent and avirulent *L. mexicana* promastigotes SLA. Shaded cells indicate a difference in regulation between virulent and avirulent parasites using the same cell line.

6) Chemokines-Cytokines-Toll like receptors network



Finger 6.3.3 The effect of the SLA stimulation on gene expression profile of Chemokines (CCLs)

Target cells were stimulated with 10 μ g virulent or avirulent *L. mexicana* SLA for 24 hours. RNA was extracted from control, infected and stimulated cells and converted to cDNA, which was used as a template for qPCR analysis. Data are the averages of least 3 independent experiments, * $P < 0.05$ was calculated using the Mann-the Whitney test. Data are presented as mean + SEM.

6.4 Discussion

The immune response to foreign antigens is modulated by complex interactions between immune host cells and invading pathogens. Like many other infectious microorganisms, *Leishmania* parasites are obligate intracellular pathogens, which can regulate/modulate any stage of the host immune response and use it for their benefit to overcome immune system defenses. Therefore, a good understanding of the mechanisms of parasite interaction with the host immune system, particularly cells targeted by the parasite, would increase the possibility of developing a potential vaccine and/or therapeutic agent. The model of virulent and avirulent parasites produced in the current study (Ali, *et. al.*, 2013) was further used to investigate and compare the ability of both sets of parasites to modulate the gene expression profile of target cells. The novelty of this investigation is the use of avirulent *L. mexicana* in parallel with its virulent counterpart which may be the best control.

To investigate the *in vitro* modulation of TLRs, ILs, and CCLs, target cells were infected with the late log phase of virulent and avirulent *L. mexicana* for 24 hours. The expression of the target genes in infected cells was evaluated using qPCR. Although the final host for *Leishmania* spp in the mammalian host is tissue macrophages, the entry of *Leishmania* promastigotes into the host blood exposes them to other types of immune cells including monocytes (Rabhi, *et. al.*, 2010). Therefore, in this study, three types of cell lines: MonoMac-6, U937 monocytes and U937 macrophages were used to compare their responses to infection with virulent and avirulent of *L. mexicana* promastigotes and their SLA.

It was difficult to draw a single clear conclusion from this study, as too many experimental variables were considered for comparison including different host cells and parasite virulency. However, TLR-1 seems to be the most affected under those experimental conditions since it showed down regulation following the infection of U937 monocytes and U937 macrophages with virulent parasite and its SLA. Variable IL-1 responses, on the other hand, were also observed to infection with virulent and avirulent parasites, this cytokine was significantly up regulated after avirulent parasite infection compared to the virulent parasite in the three used cell lines. A significant up regulation of CCL-1, CCL-2 and CCL-5 was detected in MonoMac-6 cell line infected with virulent compared to avirulent *L. mexicana*.

6) Chemokines-Cytokines-Toll like receptors network

Few studies have previously investigated the overall gene expression profile of host cells infected with different *Leishmania* parasite species using the microarrays method (Rodriguez, *et. al.*, 2004; Dogra, *et. al.*, 2007). However, little is known about the interaction between the *Leishmania* parasite and monocytes. Therefore, this may be the first study which has studied the effect of the infection with the late log phase of virulent and avirulent *L. mexicana* promastigotes and their antigens on TLRs, ILs and CCLs.

Toll like receptors (TLRs) are group of signalling receptors involved in innate immunity by recognizing the pathogen associated molecular patterns (PAMPs) presented on the cell surface and on the endosomal membrane of different types of immune cells (Gallego, *et. al.*, 2011). Pathogen recognition in the early stages of infection by TLRs leads to full activation of innate immunity. Binding TLRs to their specific set of microbial products enhances the activation of inflammatory cytokines, which work collectively to eliminate and destroy invading pathogens (Iwasaki and Medzhitov, 2004).

Many studies have reported the importance of TLRs in the way the immune system controls *Leishmania* infection. For example, TLR-4 has an important role in *L. major* control in infected Balb/c mice through its binding with MyD88 adaptor (Kropf, *et. al.*, 2004). Animal model experiments show that TLR-9 is an essential molecule in control of visceral and cutaneous Leishmaniasis (Liese, *et. al.*, 2008). A study by Shadab and Nahid (2011), illustrated the role of TLR-2 in *L. donovani* and *L. braziliensis* infection control, through the binding of TLR-2 to *Leishmania* lypophosphoglycan (LPG), which triggered the intracellular killing factors. Results of the present study have also illustrated that TLR-2 and TLR-1 are significantly down regulated after infection of U937 monocytes and U937 macrophages with virulent in comparison to avirulent *L. mexicana*. The failure of the avirulent parasite to down regulate the expression of these receptors may contribute to their killing and clearance. This is in agreement with a study by Lodge and Descoteaus (2005) who reported that the absence of TLR-2 was correlated with the increase in lesion numbers in C57BL/6 mice infected with *L. major*. Although it has been reported that TLRs are expressed on the surface of macrophages, DCs and NK cells, (Sen and Sarkar, 2005; Tuon, *et. al.*, 2008), our results have confirmed the expression of TLR-1, TLR-2, and TLR-9 on U937 and MonoMac-6 monocytes.

6) Chemokines-Cytokines-Toll like receptors network

Additionally, results presented here have shown differences in the expression pattern of TLR-9 induced by infection with *L. mexicana* promastigotes based on their virulence status and cell type. TLR-9 is normally found on the surface of endosomes and is involved in the recognition of nucleic acid bases (Katz, *et. al.*, 2006; Nicolle, *et. al.*, 2004). In the current study, although infection of U937 monocytes with virulent *L. mexicana* promastigotes has down regulated the expression of TLR-9, more down regulation was induced following infection with avirulent parasites. This might be due to the susceptibility of virulent promastigotes to the microbial killing factors secreted by monocytes when they first enter them, resulting in the degradation of virulent parasites, this may require the activation of other immune mechanisms which may favor *Leishmania* infection. In contrast, the down regulation of TLR-9 in macrophages infected with virulent parasites was significantly greater compared to the same cells infected with avirulent *L. mexicana*. This might be due to the failure of avirulent but not virulent promastigotes to produce the parasitophorous vacuoles that are needed for successful differentiation into the amastigote stage.

The effect of SLA on the gene expression profile of TLRs has not been previously investigated. Results presented in this study showed that SLA extracted from virulent parasites induced more down regulation of almost all the studied TLRs, compared to the SLA extracted from avirulent parasites. This may be due to the high expression of the virulence molecules such as GP63, LPG2, and CPC proteinase in virulent but not in avirulent *L. mexicana* promastigotes (Chapter 4). Generally, activation of TLRs signalling is essential in ILs production through activation of the NF-KB pathway (Muzio, *et. al.*, 2000). Therefore, the use of avirulent versus virulent SLA in vaccine preparation needs to be evaluated.

The vast majority of published studies have agreed that resistance to *Leishmania* parasite infection is partly dependent on the suppression of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6 and IL-12B (Muraille, *et. al.*, 2003). On the other hand, susceptibility to *Leishmania* parasite infection is associated with the up regulation of anti-inflammatory responses such as IL-4, IL-13 and TGF- β (Chaussabel, *et. al.*, 2003; Rodriguez, *et. al.*, 2004).

IL-10 is one of the anti-inflammatory cytokines produced by monocytes, macrophages, DCs and B cells (Buxbaum and Scott, 2005). According to Imada and Leonard, (2000),

6) Chemokines-Cytokines-Toll like receptors network

the biological function of IL-10 is to inhibit the production of pro-inflammatory cytokines by infected macrophages, which enhances parasite infection.

In this study (Table 6.2.3), infection of target cells with avirulent parasites caused an up regulation of IL-6 and IL-12 compared to virulent infection, a finding that highlights the role of the pro-inflammatory response in controlling *L. mexicana* infection. Although the results show that the expression of IL-10 was down regulated in MonoMac-6 and U937 macrophages following parasite infection, infection with virulent parasites induced less down regulation compared to avirulent parasite infection. This agrees with Buxbaum (2013), who reported that chronic infection with *L. mexicana*, requires IL-10, which in turn suppressed IL-12 production from macrophages, decreased IFN- γ in T cells and nitric oxide production in infected cells. Other studies have reported that in human and experimental models of CL, high levels of IL-10 production are strongly associated with non-healing forms of the disease (Buxbaum and Scott, 2005; Tripathi, *et. al.*, 2007). The results of the present study have also illustrated that infection with avirulent *L. mexicana* promastigotes caused a considerable increase in the expression of IL-1. A study by Xin, *et. al.*, (2007), highlighted the importance of IL-1 in activation of DCs and T-cell priming. This could be one of many reasons behind the failure of avirulent promastigotes to initiate the infection of inoculated Balb/c mice.

The biological role of IL-12 in controlling *Leishmania* infection has been reported through activation of Th1 type immune response and secretion of IFN- γ (Watford, *et. al.*, 2004). In experimental models, treatment of resistant mice infected with *L. major* with neutralizing anti-IL-12 antibodies caused failure of these mice to overcome their infection. Meanwhile, treatment of susceptible Balb/c mice with recombinant IL-12 has been shown to control the infection (McDowell and Sacks, 1999). Results from the current study (Table 6.2.3) show that U937 monocytes, but not MonoMac-6 cell line and U937 macrophages, highly expressed IL-12 following infection with avirulent *L. mexicana* in comparison to virulent infection. These findings reflect the multiple roles that each cytokine can play during the course of the parasite infection, using different host cells under the same experimental conditions.

It is well established that the expression or production of type one IFN- γ and type 2 INF- α , β as well as TNF- α is highly associated with resistance to *Leishmania* infection (Manna, *et. al.*, 2006; Carrillo, *et al.*, 2007). The biological role of these cytokines is

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related to activation of the innate immune response through enhancement of microbicidal activity such as iNOS (inducible nitric oxide synthase) expression and NO secretion by macrophages (Mosser and Edwards, 2005). In the present study, infection of U937 monocytes and MonoMac-6 cell line with virulent promastigotes down regulated TNF- α when compared to infection with avirulent parasites. This may inhibit NO secretion in the early stages after infection with the virulent parasite (Kavoosi, *et. al.*, 2009) thus enhancing their survival inside infected cells.

On the other hand, the up regulation of TNF- α following infection of U937 macrophages with virulent compared to avirulent parasites, may be correlated with the successful differentiation of virulent but not avirulent parasites to the more resistant amastigote stage.

The other interesting finding from this study is the expression of TGF- β , well known as an important immune regulatory cytokine. Infection of MonoMac-6 monocytes with virulent *L. mexicana* induces high expression of this cytokine, but infection with avirulent parasites induced its down regulation. Results also showed that although TGF- β was up regulated following infection with both sets of parasites, its expression was several hundred fold more in U937 macrophages infected with virulent compared to avirulent parasites, This may explain survival of virulent *L. mexicana* inside the host cells.

As mentioned above, no investigations have been reported on the effect of SLA on interleukin gene expression profile *in vitro*. In this study, results showed the regulation of cytokines by parasite infection was both cell line- and parasite-virulence dependent. Chemokines are group of polypeptides, and group CC Chemokines (which have their first two cysteines adjacent to each other) were chosen to be investigated in this study because they act on a large group of cells, such as monocytes and macrophages, but not PMNs (Moser and Willimann, 2004). In comparison, CXC Chemokines can act mainly on PMNs cells (Antoniazzi, *et. al.*, 2004). In this study, the gene expression profile of CCL-1, CCL-2, CCL-3, CCL-4, CCL-5 and CCL-22 was investigated in three different cell lines in response to infection with virulent and virulent *L. mexicana* promastigotes, and stimulation with their extracted SLA.

Since the biological role of chemokines is to recruit immune cells to the site of infection, understanding their gene regulation by modulation following *Leishmania* infection is an important tool in infection control (Ritter and Korner, 2002; Antoniazzi, *et. al.*, 2004).

6) Chemokines-Cytokines-Toll like receptors network

Results presented in Table 6.2.4 show that all tested CCLs (except CCL-5) were up regulated in U937 macrophages infected with parasites. The level of up regulation was variable depending on virulence of the parasite and the type of CCLs. For example pro-inflammatory CCL-1 chemokine was significantly up regulated following infection of U937 macrophages with virulent *L. mexicana* compared to infection with avirulent parasites. These findings were similar to those reported by Ritter, *et. al.*, (1996) who showed that patients suffering from CL exhibited higher levels of CCL-1.

Collectively, the results of this study have addressed some aspects of the complexity of the immune response to *Leishmania* spp, which is characterised by potent modulation of gene expression profile of TLRs, interleukins and chemokines in susceptible human cell lines infected with virulent or avirulent *L. mexicana* promastigotes, this clearly illustrated some of the parasites mechanisms to induce the infection. Most importantly, the results illustrate differences between infection with virulent and avirulent *L. mexicana* promastigotes using the same cell line, and these differences may help to understand how the parasite evade host immune response. Moreover, the results have clearly shown that differences were not only induced by infecting cell lines with avirulent and virulent *L. mexicana*, but also with their antigens (SLA), which may have a potential application as vaccine candidates.

Chapter 7/ General Discussion

7. 1 General Discussion

Leishmaniasis is still a major public health problem across the globe, and recently Visceral Leishmaniasis (VL), has been reported as an opportunistic infection in patients with HIV infection (Nascimento, *et. al.*, 2011). Most of the new cases of Leishmaniasis (50-75%) belong to Cutaneous Leishmaniasis (CL), therefore this has been considered the most common form of this disease (Hide, *et. al.*, 2007). CL is caused by infection with several different *Leishmania* spp, such as *L. mexicana*, *L. major*, *L. amazonensis* and *L. braziliensis*, which are transmitted to the final host by the bite of an infected sand fly. The outcome of *Leishmania* spp infection is ultimately dependent on the ability of target cells to suppress or support the transformation process from the injected promastigotes into amastigotes. Although chemotherapy drugs are available and widely used to treat *Leishmania* infection, they are compromised by unpleasant side effects and resistance. Therefore, working towards the development of a protective vaccine remains the ultimate goal to control Leishmaniasis as well as other parasitic diseases. There is so far no effective vaccine available to control the spread of Leishmaniasis, and to date the only method which has demonstrated an acceptable level of protection in humans was Leishmanisation (to mimic the natural parasite infection), *via* a deliberate inoculation of the mammalian host with attenuated *Leishmania* spp, which was banned by WHO (Laabs, *et. al.*, 2009). The use of DNA vaccine was developed as an alternative method to obtain the benefits (good immune response), and avoid the limitations of Leishmanisation. In addition, DNA itself can enhance the innate immune response *via* the activation of TLR-9 (Liu and Ulmer, 2005). It has also been illustrated that immunisation of C57BL/6 mice with CpG DNA caused activation of DCS, but not bone marrow DCs, to produce high levels of IL-2, which in turn promotes the migration of NK cells to the site of infection which, enhanced the secretion of IFN- γ , and T cell proliferation, and parasite growth in immunised mice (Laabs, *et. al.*, 2009). Wu *et. al.*, (2006) have reported that the activation of dermal DCs can also induce the production of IL-6, which in turn inhibits the accumulation of regulatory (Treg) cells at the site of infection.

The outcome of many DNA vaccine studies varied since it depended on the DNA vector, *Leishmania* antigen, and the method of delivery as well as the immunisation schedule. For example, immunisation with the *L. major* GP63 gene cloned into the

eukaryotic expression vector pCDNAI, which has the human cytomegalovirus (CMV) promoter, as a DNA vaccine induced a strong Th1 response. The immunised Balb/c mice developed significantly smaller lesions compared to the control group (immunised with empty plasmid) following *L. major* challenge (Xu and Liew, 1995). A similar finding was reported by Welker, *et. al.*, (1989), where splenocytes from Balb/c mice immunised with *L. major* GP63 cloned into pcDNA-3 plasmid produced high levels of IFN- γ but not IL-4, and also proliferated in response to *L. major* SLA, however, only 30% of immunised Balb/c mice were lesion free following a challenge with *L. major*. Another comparative study illustrated that intramuscular immunisation (I.M) with the eukaryotic expression vector pCMV3ISS encoding *L. major* LACK, PSA2, GP63, and LeIF genes as DNA vaccine, induced a significant reduction in lesion size only in mice immunised with pCMV3ISS-LACK. However, immunisation of Balb/c mice with pCMV3ISS encoding other *L. major* genes developed lesions very close in size to control mice (Ahmed, *et. al.*, 2004), a study by Dumonteil, *et. al.*, (2003) demonstrated that VR1012-GP46, VR1012-GP63 and VR1012-CPB DNA constructs partially protected Balb/c mice against infection with *L. mexicana*.

DNA vaccines also showed promising results against VL. In a study by Melby, *et. al.*, (2000) it was reported that immunisation of Balb/c mice with *L. donovani* cDNA library (extracted from the amastigote stage), cloned into pcDNA3.1 plasmid, induced a Th1 immune response and protected against challenge with *L. donovani* amastigotes. However, immunisation of dogs with multi-antigenic plasmid DNA vaccines encoding *L. infantum* KMPII, TRYP, LACK and GP63 antigens failed to protect against challenges with *L. infantum*, which also showed no humoral or cellular immune responses (Rodríguez, *et. al.*, 2007). A study by Asteal (2011) demonstrated that immunisation of Balb/c with pcRT7/CT-TOPO and pcDNA3.1/Hygro(-) plasmids encoding *L. donovani* centrin3 produced different immune responses, which may be vector dependent.

The current study thus first aimed to analyse this theory, and to do so the immunogenicity of three *Leishmania* genes constructed in three different plasmids (9 DNA constructs) was investigated. Five out of the nine *Leishmania* DNA constructs used were prepared during this study (highlighted in Table 2.2.2). The expression efficacy of the chosen plasmids was analysed by transfection in mammalian cells (Figure 3.2.7). The *Leishmania* genes used to prepare the DNA constructs are highly

conserved genes across *Leishmania* spp. In addition, the *Leishmania* GP63 gene is a known virulence factor in promastigote and amastigote stages, and which can be expressed as a secreted or membrane bound protein of the promastigote' surface (Oliver, *et. al*, 2012). *Leishmania* centrins are important for parasite proliferation and survival inside the mammalian host and were have previously been used as potential vaccine candidates (Duncan, *et. al.*, 2009, Asteal, 2011).

One of the findings of the present study is that immunisation of Balb/c mice with *L. mexicana* GP63, *L. donovani* centrin1, and *L. donovani* centrin3 genes cloned into three different plasmids induced significant immune responses. In addition, the induced immune response using these plasmids encoding *L. donovani* genes following the *in vitro* stimulation with *L. mexicana* SLA, indicates possible cross-protection against *Leishmania* species using theses DNA constructs.

The present work has also demonstrated that using *Leishmania* DNA constructs as a DNA vaccine model can induce different immune responses as has been measured using a range of different immune assays. On the other hand, the results of this study indicate that more differences in the plasmid immunogenicity were related to antigen type. For example, CTLs activity was induced in mice immunised with plasmids encoding the *L. donovani* centren3 gene compared to *L. donovani* centren1 and *L. mexicana* GP63 genes. Cell proliferation results have illustrated that plasmids encoding *L. mexicana* GP63 induced a greater proliferation rate compared to plasmids encoding *L. donovani* centrin1 and *L. donovani* centrin3 (Figure 3.3.2). The results have also shown that VR1012-L.mexgp63 induced less proliferation compared to pcRT7/CT-TOPO and pcDNA3.1/Hygro(-) plasmids encoding the same gene. Due to a lack of similar comparative investigations, there was no published data against which to compare the results of this study.

It has been also demonstrated in the present study that both Th1 and Th2 immune responses were induced, as indicated by a significant increase in IgG1 and IgG2a levels, respectively. This was in agreement with Rezvan (2007) using VR1012-L.mexgp63 and Asteal (2011), using pcRT7/CT-TOPO-Ldcen3 and pcDNA3.1/Hygro(-)-Ldcen3 in immunised Balb/c mice.

The efficacy of DNA constructs used in this study to induce the Th1 and Th2 immune responses might be appropriate for controlling *Leishmania* parasites, as has been reported by many researchers. For example, early studies demonstrated that

susceptibility and resistance to *L. major* infection were dependent on the activation of Th2 or Th1 T cell subsets, as characterised by the production of IL-4, IFN- γ , respectively. However, the use of IL-4 gene deficient mice did not confirm the role of IL-4 in disease progression (Kopf, *et. al.*, 1996; Noben-Trauth, *et. al.*, 1996; Kropf, *et. al.*, 2003). On the other hand, transgenic IL-12 Balb/c mice developed non-healing lesions following *L. major* challenge, despite high levels of IL-12 (Alexander and Bryson, 2005). These contradictory reports indicate that other regulatory cytokines may be involved in *Leishmania* parasite pathogenicity, such as IL-13 (Mohrs, *et. al.*, 1999). Another study reported that IL-13 transgenic resistant C57BL/6 mice developed non-healing lesions following *L. major* infection (Matthews, *et. al.*, 2000). In addition to the *L. mexicana* LACK antigen, cysteine proteases expressed in promastigotes and amastigote were also found to be virulence factors (Matthews, *et. al.*, 2004).

It has been reported that the sensitivity of Balb/c mice to *L. major* infection was due to the expression of LACK antigen, a factor that activates IL-4 production through binding with $\nu\beta 4V\alpha 8$ CD4⁺ T cells, and suppresses IL-12 production (Launois, *et. al.*, 1995). Similar to that of *L. major* infection, IL-4 cytokine was also found to play a major role in non-healing lesions developed in Balb/c mice infected with *L. mexicana* promastigotes, compared to the contribution of IL-10 and IL-13 (Padigel, *et. al.*, 2003). Other studies by Alexander, *et. al.*, (1998) and Pollock, *et. al.*, (2003) have reported that IL-4 was induced due to the presence of the virulence factor Cathepsin-L in mice infected with *L. mexicana*. Different scenarios of the role of the Th2 response during *L. donovani* infection using genetically modified mice have been recorded. The results showed that IL-4 deficient B6/129 and Balb/c mice were more susceptible to *L. donovani* infection, compared to wild types (Stäger, *et. al.*, 2003; Stäger, *et. al.*, 2003). Furthermore, cured Balb/c mice have been characterised by the presence of Th1 and Th2 related cytokines (Engwerda, *et. al.*, 1998).

The immunogenicity of the constructed plasmids was further analysed by a novel assay, which has been developed during the course of this study (section 3.3-6). This assay clearly shows that immunisation with these constructs caused dramatic effects on the parasite's natural life cycle in Balb/c BM-DMs. This was characterized by the inability of the amastigote stage harvested from macrophages generated from immunised mice to transform into the promastigote stage. To the best of our knowledge, this is the first study designed to compare the immune response induced by immunisation with

different *Leishmania* DNA constructs encoding the same gene to evaluate the vector effect on the immune response. However, further investigations are required, including live parasite challenge experiments, to assess the efficacy of protection against live parasites.

In this study, the growth characteristics of *L. mexicana* have been investigated, and the effect of *in vitro* culturing on *L. mexicana* promastigote infectivity was compared at different passage numbers (P1, P7 and P20). The loss of *L. mexicana* virulence has been reported *in vitro* and *in vivo* (Chapter 4), which has been published (Ali, *et. al.*, 2013).

Loss of parasite infectivity by long *in vitro* culture has been previously reported (Wozencraft and Blackwell, 1987). In this study the effect of long-term culture on different aspects of *L. mexicana* promastigote growth, such as parasite infectivity *in vitro* and *in vivo* and virulence associated gene expression was examined. For example, *L. mexicana* virulence associated genes (listed in table 2.2.4) were significantly down regulated in avirulent promastigotes, which may explain the failure of the parasite at passage 20 to initiate the infection in Balb/c mice, and also the failure of amastigote transformation in infected host cells. In addition, for the first time, the correlation between parasite body size and virulence loss is reported. This model of virulent (Passage 1), and avirulent (Passage 20) *L. mexicana* has been extensively investigated in the current study, and many differences between both types of parasite have been reported (Ali, *et. al.*, 2013).

Attenuated parasites have been produced using various methods, for example by irradiation, chemical mutagenesis or culturing under antibiotic pressure, and recently, by deleting specific genes from the parasite genome. In particular attenuation using dihydrofolate reductase-thymidylate synthetase (DHFR-TS), in long term survival in host cells (Titus, *et. al.*, 1995; Veras, *et. al.*, 1999).

Early studies reported limited protection against infection with *L. major* and *L. amazonensis* following immunisation of a mouse model with DHFR-TS knockout *L. major* (Titus and Veras 1995). However, these parasites failed to induce protection in monkeys (Amaral, 2002). Although the virulence genes knockout method has been widely used to produce attenuated parasites (by deleting particular genes such as cysteine proteinase or LPG1), these parasites were still able to induce the infection (Ryan, 1993; Huango and Turo 1993). In another study, LPG2 knockout *L. major*

(responsible for encoding an enzyme involved in the transport of GDP-mannose to the Golgi apparatus) was able to survive in Balb/c mice and provided protection against homologous infection (Uzonna, 2002). Unlike the protection induced by other vaccines, Kedzierski, and Zhu (2006) suggested the involvement of different mechanisms in *Leishmania* infection control, since the protection induced by attenuated parasites was not related to the Th1 response and IFN- γ production.

It has been reported that a successful Leishmaniasis vaccine is based on a continuous presence of *Leishmania* antigens inside the host which can be achieved by vaccination with attenuated parasites or subunit vaccines (Paoletti and McInnes, 1999). In addition, that attenuated parasites can be taken up by the host cells in the same way as virulent parasites and persist inside them without the induction of the infection (Kedzierski, and Zhu, 2006). In this study, the infectivity of avirulent *L. mexicana* (P20) was evaluated. Although the percentage of infected cells with avirulent parasites was very low compared to infection with virulent parasites after 2 hours, 48 hours after infection many avirulent parasites were observed inside the target cells, indicating their ability to survive inside host cells. In addition, these promastigotes were unable to transform into the pathogenic amastigote stage. Furthermore, the growth of avirulent *L. mexicana* promastigotes was inhibited in conditioned medium derived from *L. mexicana* infected host cells illustrating the inability of these parasites to proliferate in the presence of secreted material from infected target cells. These findings indicate poor infectivity of avirulent (P20) compared to virulent *L. mexicana* (P1). Since, the results also show the down regulation of virulent genes, which contribute to parasite pathogenicity, avirulent parasites produced in this study, might be a good attenuated vaccine candidate as a model against *Leishmania* parasite infection.

It has been reported that the number of parasites per ml can define the stationary phase of *Leishmania* growth curve. This number was considered to be above 2.5×10^7 depending on *Leishmania* species, type of medium and growth conditions (Fritsche, *et. al.*, 2007). The results of this study have demonstrated that *L. mexicana* promastigote numbers (cultured in *Drosophila* Schneider medium supplemented with 10% v/v HIFCS at 25°C) dramatically decreased after reaching a concentration of 6×10^7 per ml. There was no recognised stationary phase, which was in agreement with Fritsche *et. al.*, (2007). The decreased number of *Leishmania* promastigotes is due to the depletion of nutrients, the increase of waste metabolites, and presence or absence of oxygen

(Iqbal, *et. al.*, 2006; Fritsche, *et. al.*, 2007). The *L. mexicana* growth conditions used in this study gave no stationary phase compared to their growth in the presence of O₂, where the stationary phase can be seen in the growth curve (Appendix 14). As a result, the stationary phase in this study was referred to as the late log phase which has the same properties as the stationary phase (Figure 5.2.5, A, B, C).

The virulency of the parasite following growth in two different conditions was compared and the results clearly show that growth was better in the absence of O₂ which was associated with high expression of virulence associated genes, compared to parasite grown in the presence of O₂ (Appendix 15).

Investigations carried out in the current study also extended to address the effect of mouse host living conditions on the virulency of the parasites. It is reported here that the virulency of *L. mexicana* promastigotes (measured by expression of virulence associated genes by qPCR), which was generated in mice living in a Barrier Unit (under completely sterile conditions), was significantly less compared to *L. mexicana* generated in mice kept in a conventional unit, where the mice are exposed to normal (non-sterile) conditions (Appendix 12).

Although a lot is known about the interaction of the host with *Leishmania* parasites, most of the available data are obtained from murine models, and there is still a lack of research assessing human-specific responses to *Leishmania* infection (Stockdale and Newton, 2013). In this study, three human cell lines: U937 monocytes, U937 macrophages, and MonoMac-6 monocyte, were used as host cells to investigate their interaction with virulent and avirulent *L. mexicana* promastigotes. Intracellular pathogens such as the *Leishmania* parasite can subvert the host's immune responses using various mechanisms, one of which is the down regulation of MHC class I. This phenomenon was investigated in the current study, using both virulent and avirulent *L. mexicana* promastigotes (Chapter 5).

Presentation of the parasite antigens by MHC class I is essential for the recognition and killing of infected cells by CD8⁺ T cells (Lieberman, 2003). Down regulation of MHC class I molecules keeps infected cells undetectable by effector cells which may help the parasites to survive and successfully differentiate into the more resistant intracellular amastigote stage (Hansen and Bouvier, 2009).

This study has focused on the effect of infection with *L. mexicana* promastigotes taken at different growth stages *in vitro* on the expression of MHC class I, using susceptible

human cell lines. The infectivity of *L. mexicana* promastigotes varies from phase to phase during its cell cycle, and the growth stages.

Interestingly, infection of target cells with the more infectious mid log or late log stages of *L. mexicana* promastigotes has significantly less effect on MHC class I expression compared to the less infectious early log stage, using a ratio of infection 1:10 (cell: parasites). In addition, no differences between virulent and avirulent *L. mexicana* promastigotes on MHC class I expression were noted in either cell lines used in this study. It is well documented that, during their growth cycle *Leishmania* promastigotes go through a series of maturation steps, including differences in morphology and motility (Wozencraft and Blackwell 1987). Investigations on the infectivity of *L. mexicana* promastigote during the less effective early log stage compared to late log growth stage revealed that the parasite infectivity was greater in the late log phase. Therefore, the parasites during the late log phase were considered as metacyclic promastigotes for further investigation. Infection of the host cells with a ratio of 1: 20 (cell: parasites) significantly down regulated MHC class I expression following infection with both virulent and avirulent parasites.

Although these findings suggest that MHC class I expression was infection-dose dependent, however, the expression of MHC class I was restored following the treatment of infected cells with an anti-parasite agent (Fungizone). This was in agreement with an early study by Wolfram, *et. al.*, (1995) who demonstrated that infected BM-DMs efficiently presented *L. mexicana* antigen when the infected cells were treated with L-leucine methylester which caused parasite killing and degradation, so T cells were strongly stimulated.

Intracellular pathogens such as viruses have developed various mechanisms to establish the infection, such as interference with biosynthesis, assembly, transportation, localization and down regulation of MHC class II and MHC class I (Yewdel and Hill, 2002; Peterlin and Trono, 2003). Since *Leishmania* parasites are also intracellular pathogens, they are likely to follow the same strategies as viruses in order to evade immune system defences. In addition to the direct effect of down regulation of MHC class I or class II expression on the interaction of T cells with APCs, there is an inhibition effect on leishmanicidal cytokines (Green, *et. al.*, 1990; Kaye, 1995). This emphasised the importance of antigen presentation in the control of parasite infection, and so a number of studies investigated the presentation of *Leishmania* antigen.

However, the results from these studies were mostly conflicting and difficult to interpret.

For example, specific CD8⁺ T cells specifically proliferated in a response to antigens presented *via* MHC class I molecules following infection with *L. amazonensis* (Kima, *et. al.*, 1997). In contrast, a study by Wolfram, *et. al.*, (1995) demonstrated that infected bone marrow-derived macrophages failed to stimulate the proliferation of specific T helper 1 (Th1) cell line which had been generated against *L. mexicana* cysteine proteinases, when they were induced to express MHC class II by interferon IFN- γ , and that there was no effect on parasite viability. A study reported that antigen presentation by Balb/c BM-DMs infected with *L. donovani* has a limited effect on T cell response (Meier, *et. al.*, 2003). Consequently, in the current study, different aspects have been considered, such as the effect of passage number, *L. mexicana* growth stages, and infection doses on MHC class I expression, which can cause considerable variation in the obtained results.

Parasitophorous vacuoles are important for the parasites. In these, the parasites can create their own environment and cover themselves with endoplasmic membrane within a closed vesicle that is advantageous to transformation of the susceptible promastigote stage to the more resistant amastigote stage (Gregory, *et. al.*, 2008). One possible explanation for losing infectivity of avirulent *L. mexicana* might be due to the failure of promastigotes to produce the PV, which ultimately leads to their failure to differentiate into the amastigote stage (Ali, *et. al.*, 2013). In addition, qPCR results in this study showed the absence of phagosome maturation (Rab7 and Rab9) in target cells infected with virulent *L. mexicana* promastigotes, whereas these markers were highly expressed in cells infected with avirulent parasites.

To evaluate the modulation induced by *L. mexicana* or their antigens on innate and adaptive immune responses, susceptible human cell lines were infected with virulent and avirulent parasites, or stimulated with parasite antigens extracted from virulent and avirulent parasites, and the gene expression profile of some TLRs, CCLs, and ILs were investigated (Chapter 6).

Many studies have reported the importance of understanding the early events of immune response to the parasite infection in order to control Leishmaniasis (Teixeira, *et. al.*, 2006; Tuon, *et. al.*, 2008; Gallego, *et. al.*, 2011). The early immune response to foreign antigens involves recognition by TLRs, this will trigger the release of

chemokines, cytokines and recruitment of other cell types to the infection site. These three key elements (TLRs, CCLs, and ILs) have mechanisms in place to regulate each other (reviewed by Teixeira, *et. al.*, 2006). Although there is a general agreement that the Th1 immune response is associated with *L. major* infection control, and Th2 is associated with disease progression in susceptible Balb/c mice, other work has also reported the role of both types of immune response in *L. donovani* infection (Palatnik, *et. al.*, 2008). Interestingly, the immune response can also contribute to disease progression (Gonzalez, *et. al.*, 2013). For example in CL, where the parasites are localized in skin macrophages, the inflammatory response can also help parasite survival in infected cells (Terabe, *et. al.*, 2000). This confirms the fact that *Leishmania*-host cell interaction is poorly understood. Therefore, a comprehensive understanding of the possible pathways that the parasite follows during the infection, and the regulatory mechanisms of the immune system is essential, not only for vaccine development, but also for the development of *Leishmania* immunotherapy.

To develop a model which enables understanding of the interaction network and possible immune response induced by parasite infection, susceptible human cell lines (U937 monocytes, U937 macrophages, MonoMac-6 monocytes) were infected with virulent and avirulent *L. mexicana* or stimulated with their antigens for 24 hours, followed by RNA extraction and cDNA synthesis. The same products were used to analyse the expression profile of TLRs, CCLs, and ILs, and to the best of our knowledge, no similar approaches have been reported so far to investigate host cell-parasite interaction. In addition, using virulent and avirulent *L. mexicana* promastigotes or their antigens (SLA) to infect or stimulate the target cells, would help to understand:

- I) How the virulent parasites evade the immune response.
- II) How the immune response controls infection through the response of target cells to avirulent parasite.
- III) How parasite antigens stimulate immunity.

The parasite antigens have a similar pattern of effects on TLRs, which reflect the fact that these parasites after 24 hours of infection can suppress the role of TLRs, which in turn inhibits the activation of other immune response mechanisms. More importantly, different time points should also be considered since parasites have different impacts on target cells. For example TLR-1, TLR-2, and TLR-9 were down regulated 24 hours

after infection with the virulent parasite but first significantly up regulated 2 hours post infection with the same parasites (data not shown).

Generally, these results are in agreement with other findings. For example, it has been reported by Chandra and Naik (2008) that infection of THP-1 monocytes, and monocytes derived from human peripheral blood with *L. donovani*, caused down regulation gene expression profile of TLR-2 (which was down regulated by virulent infection in this study) and led to IL-10 production. Another study has demonstrated that infection of susceptible Balb/c mice with *L. donovani* caused down regulation of TLR-2, which in turn suppressed the production of IL-12 (Bhattacharya, *et. al.*, 2010). Pro-inflammatory cytokines such as IL-1, IL-6 were up regulated as a result of avirulent infection, and published data by Ali *et. al.*, (2013) showed IL-1 was highly expressed after 2 hours infection with virulent parasites, compared to infection with avirulent parasites.

The scenario followed by virulent parasite can be explained as follows:

When *L. mexicana* first interacts with host cells, they activate their immune mechanisms, which causes recruitment of many cells to the site of infection, which allows the parasite to enter and infect as many as possible. In 24 hours, once the parasites are inside the host cells and transformed into the more resistant stage (amastigotes), they suppress the immune responses.

Activation and suppression of tested TLRs, ILs, and CCLs in infected human cell lines infected with virulent and avirulent *L. mexicana* promastigotes for 24 hours, or stimulation with their antigens, were complex and depend on the state of the cell type, the parasite virulence status, and the time point at which events were measured. These findings have addressed an important fact, which is the diversity and complexity of the immune response to *Leishmania* parasites and it may one of reasons behind a failure to develop an effective vaccine to *Leishmania* parasites.

7.2 Future work

The data presented in this study have addressed some aspects of *Leishmania* DNA vaccines and interaction of *L. mexicana* promastigotes with host cells. Further studies are required to address the many questions generated:

- 1) The data presented demonstrates the ability of *Leishmania* DNA constructs to stimulate both Th1 and Th2 immunity, along with high levels of IFN γ secretion and splenocyte proliferation in immunised Balb/c mice. The ability of these constructs to protect *in vivo* against challenges with live *L. mexicana* needs further investigation.
- 2) Since results of this study have demonstrated the immunogenicity of *L. mexicana* GP63, *L. donovani* centrin1 and centrin3 genes individually, it would be useful to sub clone them in one plasmid, and then test their immunogenicity.
- 3) More investigations are required to evaluate the interesting findings of the survival assay, such as inoculation of Balb/c mice with amastigotes obtained from immunised mice, and monitoring their ability to induce infection.
- 4) This study has shown the inability of avirulent *L. mexicana* promastigotes to induce infection. Therefore, these parasites or other attenuated species (using the same methods) might be promising Leishmaniasis vaccine candidates.
- 5) Results also have demonstrated the presence of growth factors that enhance or inhibit the growth of virulent and avirulent *L. mexicana*. Secreted by infected host cells, the content of these growth factors needs to be identified using mass spectrometry.
- 6) The contradictory findings related to MHC class I expression following parasite infection were evaluated and controlled in this study, so the effect of other *Leishmania* species on MHC class I expression needs to be investigated and compared.
- 7) Further understanding of the role of TLRs, ILs, and CCLs in *Leishmania* parasite-host cell interaction mechanisms is required. This would show which changes in the immune regulatory mechanism are responsible for allowing promastigotes to interact with and enter the host cells, (short time infection), and which of them are required for amastigote maintenance and proliferation (long time infection). In addition, these molecular changes need to be confirmed

at protein levels by flow cytometry analysis and immunofluorescence staining, this will illustrate if there is a direct relationship between gene expression and protein secretion in response to *Leishmania* infection.

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Appendix

Appendix

```

sequence1 CGTCTGAGAGTCGGAACCTGTGGTATAACAGCATACAATACTTCGTTGGCGACGTACTGG
sequence2 -----TGTGGTATAACAGCATACAATACTTCGTTGGCGACGTACTGG
*****

sequence1 CAGTACTTCACCAATGCGTCCCTCGGGGGCTACTCGCCATTCTTGGA CTACTGCCC GTTT
sequence2 CAGTACTTCACCAATGCGTCCCTCGGGGGCTACTCGCCATTCTTGGA CTACTGCCC GTTT
*****

sequence1 GTTGTTGGCTACAGGAATGGCTCGTGCAATCAGGATGCGTCGACGACACCGGACCTTCTC
sequence2 GTTGTTGGCTACAGGAATGGCTCGTGCAATCAGGATGCGTCGACGACACCGGACCTTCTC
*****

sequence1 GCTGCGTTCAACGTCTTCTCCGAGGCCGCGCGGTGCATCGATGGCGCCTTCACGCCGAAG
sequence2 GCTGCGTTCAACGTCTTCTCCGAGGCCGCGCGGTGCATCGATGGCGCCTTCACGCCGAAG
*****

sequence1 AACAGAACCGCTGCGGATGGATACTACACCGCCCTGTGCGCCAACGTGAAGTGCACGACG
sequence2 AACAGAACCGCTGCGGATGGATACTACACCGCCCTGTGCGCCAACGTGAAGTGCACGACG
*****

sequence1 GCCACGCGCACGTACAGCGTCCAGGTGCGCGGCAGCAACGGCTACGCCAACTGCACGCCG
sequence2 GCCACGCGCACGTACAGCGTCCAGGTGCGCGGCAGCAACGGCTACGCCAACTGCACGCCG
*****

sequence1 GGCCTCAGAGTTAAGTTGAGCAGCGTGAGCGACGCCTTCGAGAAGGGCGGCTACGTCACG
sequence2 GGCCTCAGAGTTAAGTTGAGCAGCGTGAGCGACGCCTTCGAGAAGGGCGGCTACGTCACG
*****

sequence1 TGCCCGCCGTACGTGGAGGTGTGCCAGGGCAACGTCAAAGCTGCCAAGGACTTTGCAGGC
sequence2 TGCCCGCCGTACGTGGAGGTGTGCCAGGGCAACGTCAAAGCTGCCAAGGACTTTGCAGGC
*****

sequence1 GACACCGACAGCTCCAGCAGCGCCGATGACGCTGCCGACAAAGAGGCGATGCAGCGGTGG
sequence2 GACACCGACAGCTCCAGCAGCGCCGATGACGCTGCCGACAAAGAGGCGATGCAGCGGTGG
*****

sequence1 AGTGACAGGATGGCCGCCTTGGCTACTGCGACGACGCTGCTGCTAGGAATGGTGCTCTCT
sequence2 AGTGACAGGATGGCCGCCTTGGCTACTGCGACGACGCTGCTGCTAGGAATGGTGCTCTCT
*****

sequence1 CTCATGGCACTCCTCGTGGTGCGGCTACTCCTTACCAGCTCCCCCTGGTGCTGCTGCAGA
sequence2 CTCATGGCACTCCTCGTGGTGCGGCTACTCCTTACCAGCTCCCCCTGGTGCTGCTGCAGA
*****

```

Appendix 1- The alignment of sequenced *L. mexicana* gp63 gene

Sequence alignment of the region of *L. mexicana* gp63 gene that was sequenced using primer 1 and 2 (Table 2.2.4). Sequence 1 is the standard *L. mexicana* gp63 gene, and Sequence 2 corresponds to Source BioScience results.

```

sequence1 -----CGCCGACGGC
sequence2 ATGGCTGCGCTGACGGATGAACAGATTGCGGAGGCCTTCAACCTCTTCGACGCCGACGGC
          *****

sequence1 TCTGGCGCTATCGACGCGGAGGAGATGGCGCTAGCGATGAAGGGTCTCGGGTTCGGTGAC
sequence2 TCTGGCGCTATCGACGCGGAGGAGATGGCGCTAGCGATGAAGGGTCTCGGGTTCGGTGAC
          *****

sequence1 CTGTCGCGCGACGAGGTGGAGCGCATTATCCGCTCTATGCACACAGACTCGAACGGTCTG
sequence2 CTGTCGCGCGACGAGGTGGAGCGCATTATCCGCTCTATGCACACAGACTCGAACGGTCTG
          *****

sequence1 GTGGCGTACGGCGAGTTTGAGGCCATGGTCAAGTCGCGCATGGCGCAGAAGGACTCGCCG
sequence2 GTGGCGTACGGCGAGTTTGAGGCCATGGTCAAGTCGCGCATGGCGCAGAAGGACTCGCCG
          *****

sequence1 GAGGAGATCCTAAAGGCCTTTCAGCTCTTCGACCTCGATAAGAAAGGCAAAATCTCCTTT
sequence2 GAGGAGATCCTAAAGGCCTTTCAGCTCTTCGACCTCGATAAGAAAGGCAAAATCTCCTTT
          *****

sequence1 GCGAACTTGAAGGAGGTTGCGAAACTGCTGGGTGAGAACCCCGGCGACGATGTGCTGAAG
sequence2 GCGAACTTGAAGGAGGTTGCGAAACTGCTGGGTGAGAACCCCGGCGACGATGTGCTGAAG
          *****

sequence1 GAGATGATCGCCGAGGCCGATGAGGACGGTGATGGCGAGGTGTCCTTCGAGGAGTTCAAG
sequence2 GAGATGATCGCCGAGGCCGATGAGGACGGTGATGGCGAGGTGTCCTTCGAGGAGTTCAAG
          *****

sequence1 AGCGTGATGCTGCACATGCGTGGAAAGTAGGAGAGATCTCAAGGGCGAATTCCAGCACAC
sequence2 AGCGTGATGCTGCACATGCGTGGAAAGTAG-----
          *****

```

Appendix 2- The alignment of sequenced *L. donovani* centrin1 gene

Sequence alignment of the region of *L. donovani* centrin1 gene that was sequenced using primer 5 (Table 2.2.2). Sequence 1 is the standard *L. donovani* centrin1 gene, sequence 2 corresponds to the sequence produced by MWG-Biotech.

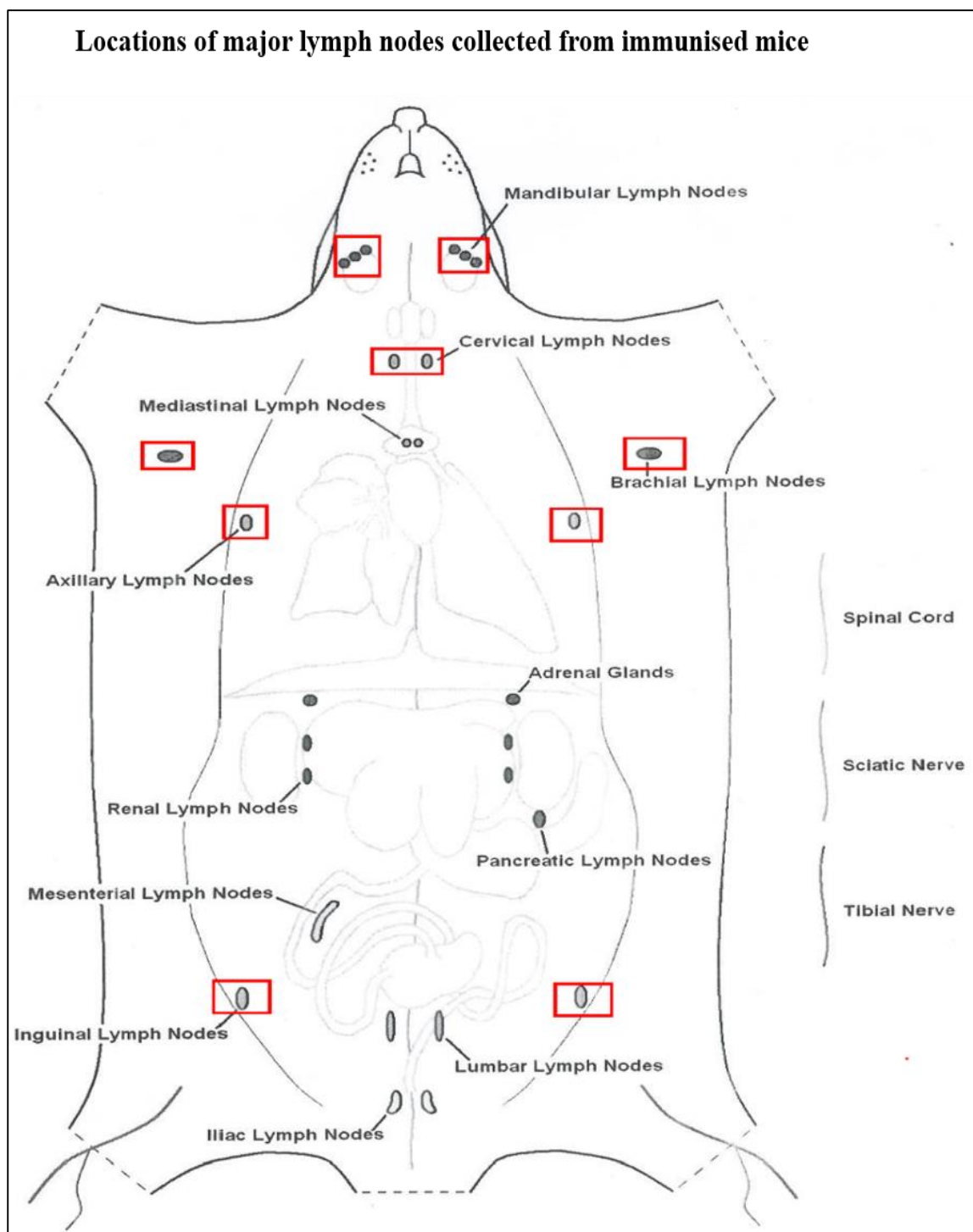
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sequence1 ATGAACATCACTAGTCGCACATCGGGGCCGCTGCGCACCACTGCGCCGGCGGCATCAGCG
sequence2 -----CTGCGCACCACTGCGCCGGCGGCATCAGCG
*****
sequence1 CCGTCCGCGGCAGCGCGCCGTCGCTTCCAGCTTACGGAGGAACAGCGCCAGGAGATCCGA
sequence2 CCGTCCGCGGCAGCGCGCCGTCGCTTCCAGCTTACGGAGGAACAGCGCCAGGAGATCCGA
*****
sequence1 GAGGCATTTCGAGCTGTTTCGACTCGGATAAGAACGGACTCATCGATGTGCATGAGATGAAG
sequence2 GAGGCATTTCGAGCTGTTTCGACTCGGATAAGAACGGACTCATCGATGTGCATGAGATGAAG
*****
sequence1 GTCAGCATGCGAGCACTTGGCTTTGATGCAAAACGGGAGGAGGTGCTGCAGCTCATGCAG
sequence2 GTCAGCATGCGAGCACTTGGCTTTGATGCAAAACGGGAGGAGGTGCTGCAGCTCATGCAG
*****
sequence1 GACTGCGCTGCCCGGGACCAGAACAATCAGCCGCTTATGGACTTACCGGGCTTCACAGAT
sequence2 GACTGCGCTGCCCGGGACCAGAACAATCAGCCGCTTATGGACTTACCGGGCTTCACAGAT
*****
sequence1 ATCATGACGACAAGTTTTCGCGCAGCGCGATCCTCGGCAGGAGATGGTGAAGGCGTTTCAG
sequence2 ATCATGACGACAAGTTTTCGCGCAGCGCGATCCTCGGCAGGAGATGGTGAAGGCGTTTCAG
*****
sequence1 CTGTTTGACGAGAACAATACCGGCAAAATCTCCCTTCGCTCGCTGCGTCGTGTGGCGCGG
sequence2 CTGTTTGACGAGAACAATACCGGCAAAATCTCCCTTCGCTCGCTGCGTCGTGTGGCGCGG
*****
sequence1 GAACTGGGCGAGAACATGAGCGACGAAGAGCTGCAGGCAATGATTGACGAGTTTGACGTA
sequence2 GAACTGGGCGAGAACATGAGCGACGAAGAGCTGCAGGCAATGATTGACGAGTTTGACGTA
*****
sequence1 GATCAAGATGGCGAGATCAACCTAGAAGAGTTTCTTGCCATTATGCTAGAGGAGGACGAC
sequence2 GATCAAGATGGCGAGATCAACCTAGAAGAGTTTCTTGCCATTATGCTAGAGGAGGACGAC
*****
sequence1 TAC-----
sequence2 TACAGAAATC
***

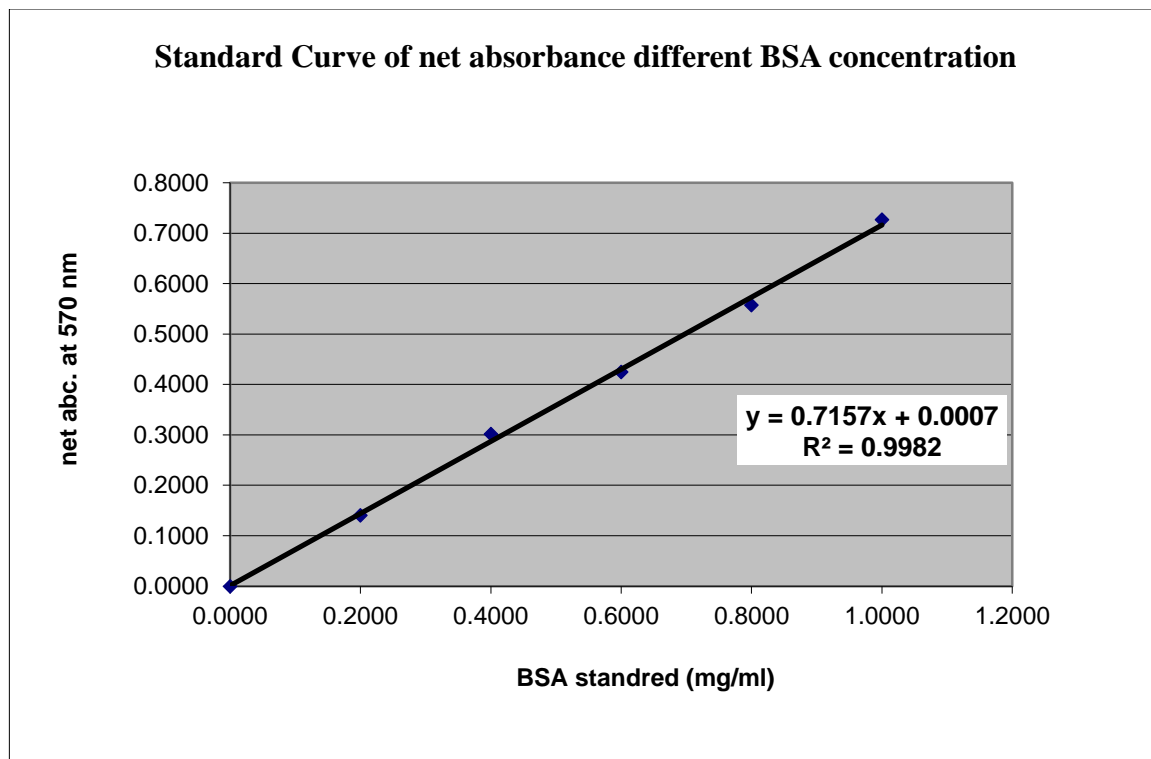
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Appendix 3- The alignment of sequenced *L. donovani* centrin3 gene

Sequence alignment of the region of *L. donovani* centrin3 gene that was sequenced using primer 3 (Table 2.2.2). Sequence 1 corresponds to the sequence produced by MWG-Biotech. Sequence 2 is the standard *L. donovani* centrin3 gene.

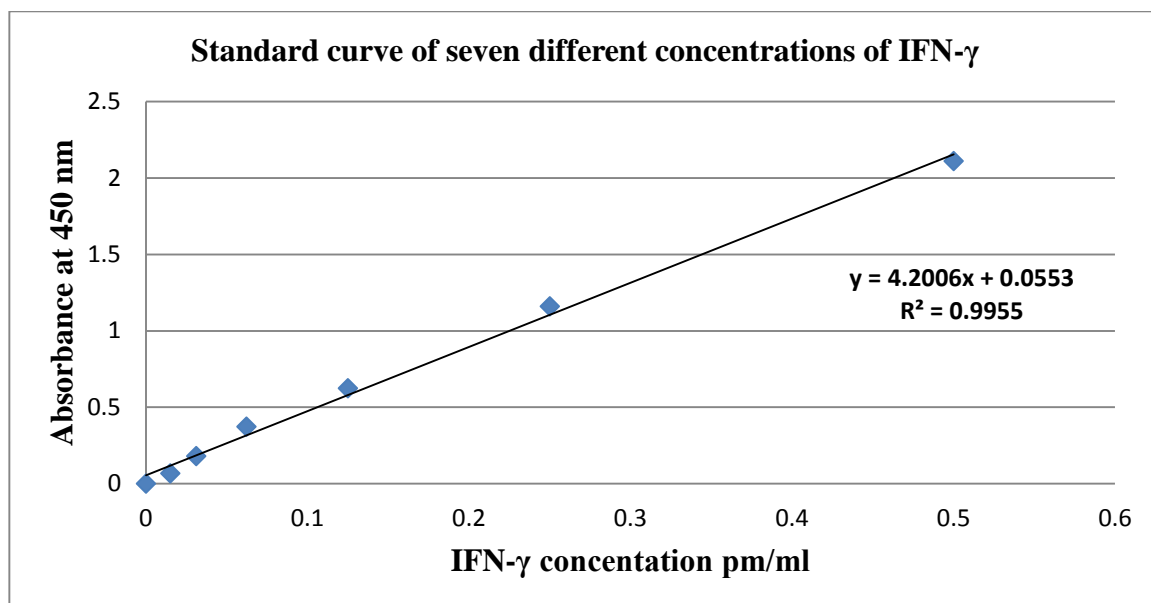


Appendix 4- Scheme illustrated the lymph nodes (marked red square) which were collected from control and immunised mice.

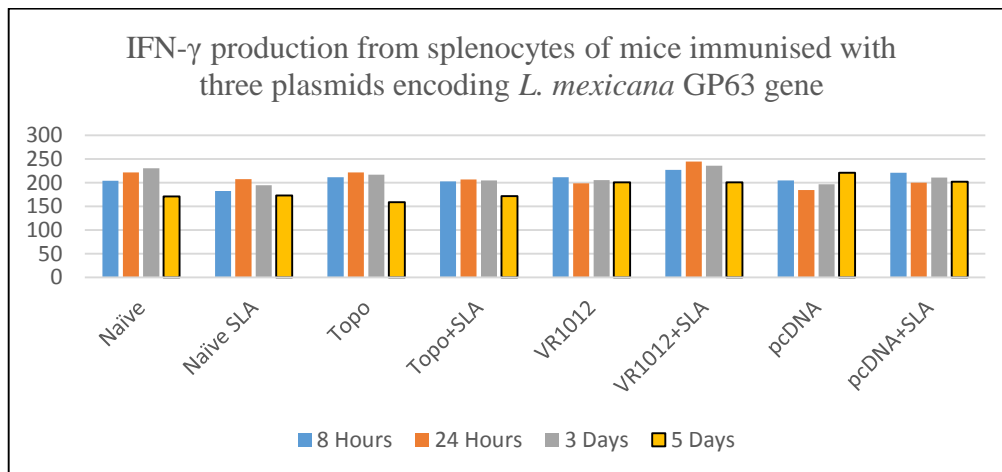
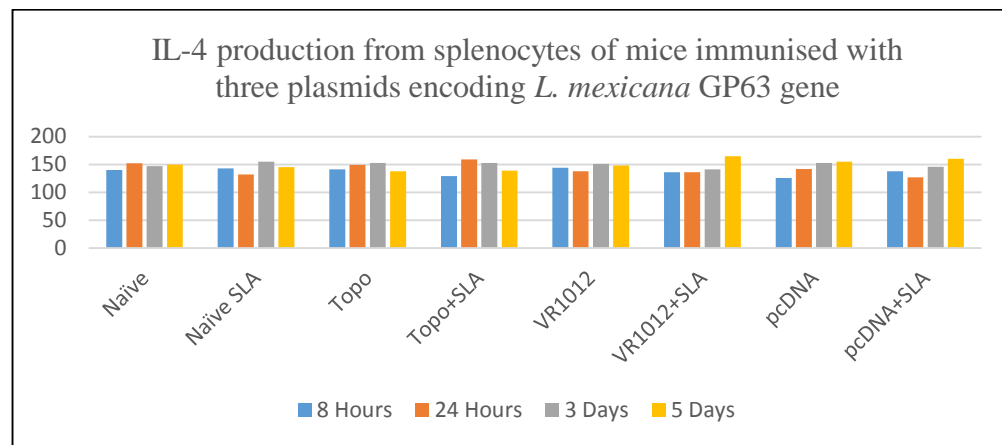
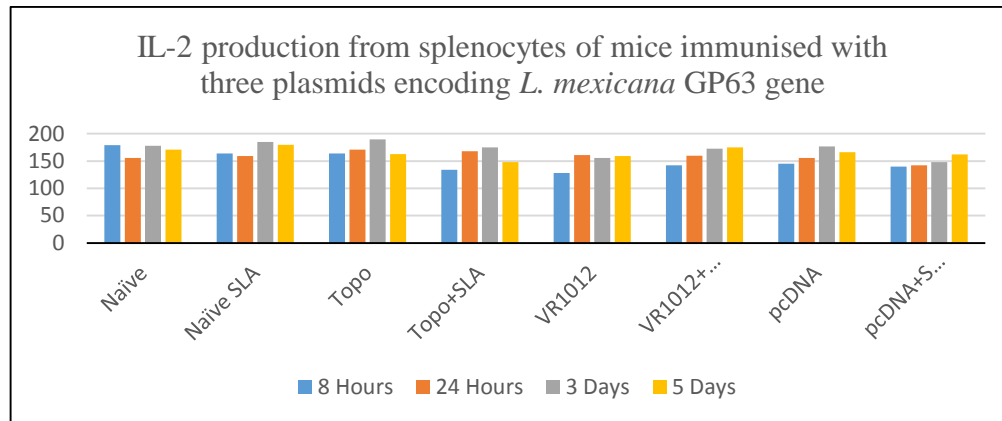


Appendix 5- Standard curves of BSA used to determine SLA concentrations.

A standard curve was produced for each experiment

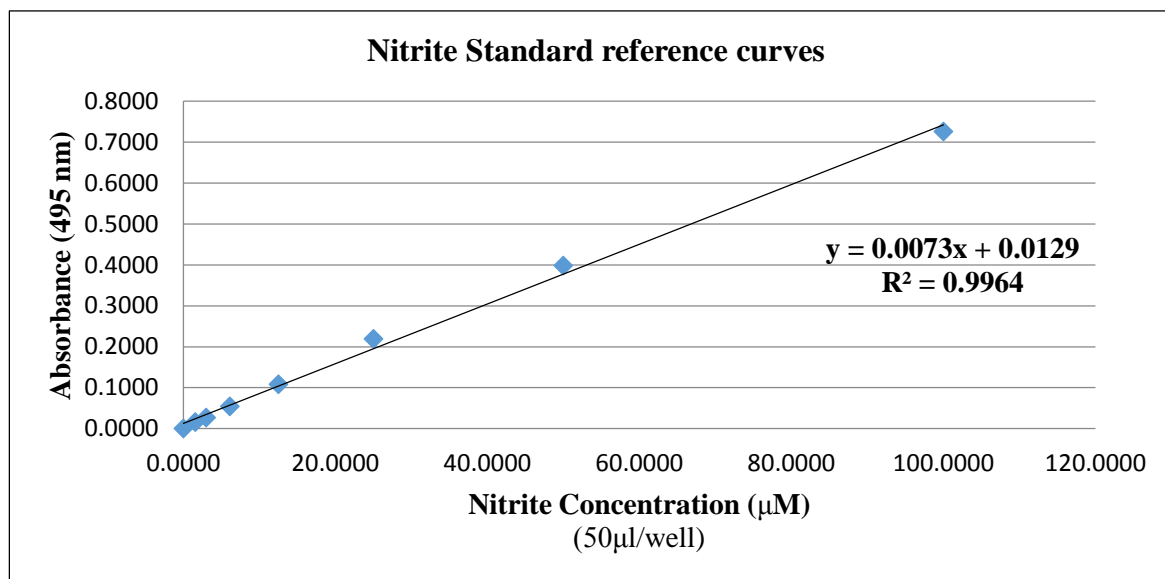


Appendix 6- Standard curve used to determine the level of IFN- γ
(Standard curve was performed to each plate)



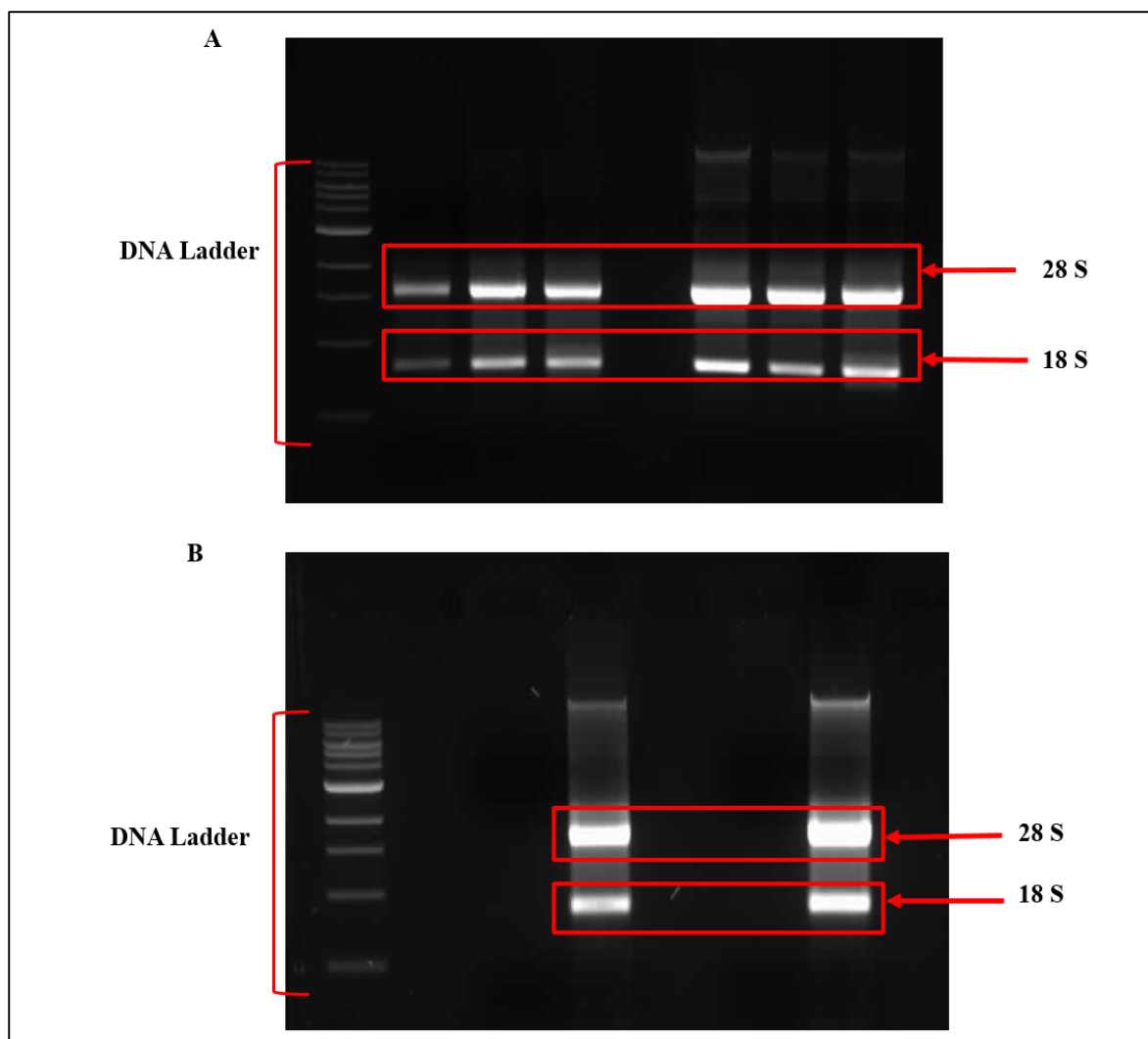
Appendix 7- cytokines secreted from splenocytes of immunised mice

Splenocytes from controls and immunised mice were cultured at a concentration of one million cells per ml in 24 well plates and stimulated with *L. mexicana* SLA. Plates were incubated at 37°C, and supernatants were collected at 8 hours, 24 hours, 3 days, and 5 days. The level of secreted cytokines was measured using ELISA method according to the manufacturer's protocol. Results showed there was no differences in the cytokine production when the cells from control and immunised mice were cultured in the presence or absence of SLA.



Appendix 8- Nitrite Standard reference curves which used to evaluated the NO concentration

To evaluate the NO concentration in the serum of infected cells, a standard curve was performed for each experiment according to the manufacture's protocol as described in the material a method section



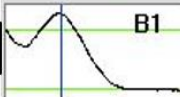
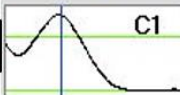

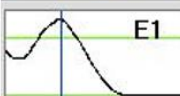
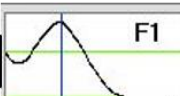


Appendix 9- Quality of RNA analysis using agarose gel

3ul of extracted RNA (A from human cell lines, B from *L. mexicana* promastigotes) was run into 1.5% agarose gel in one X TAE buffer for 50 minutes at voltage 80. The upper band is 28S, and the lower band is 18S

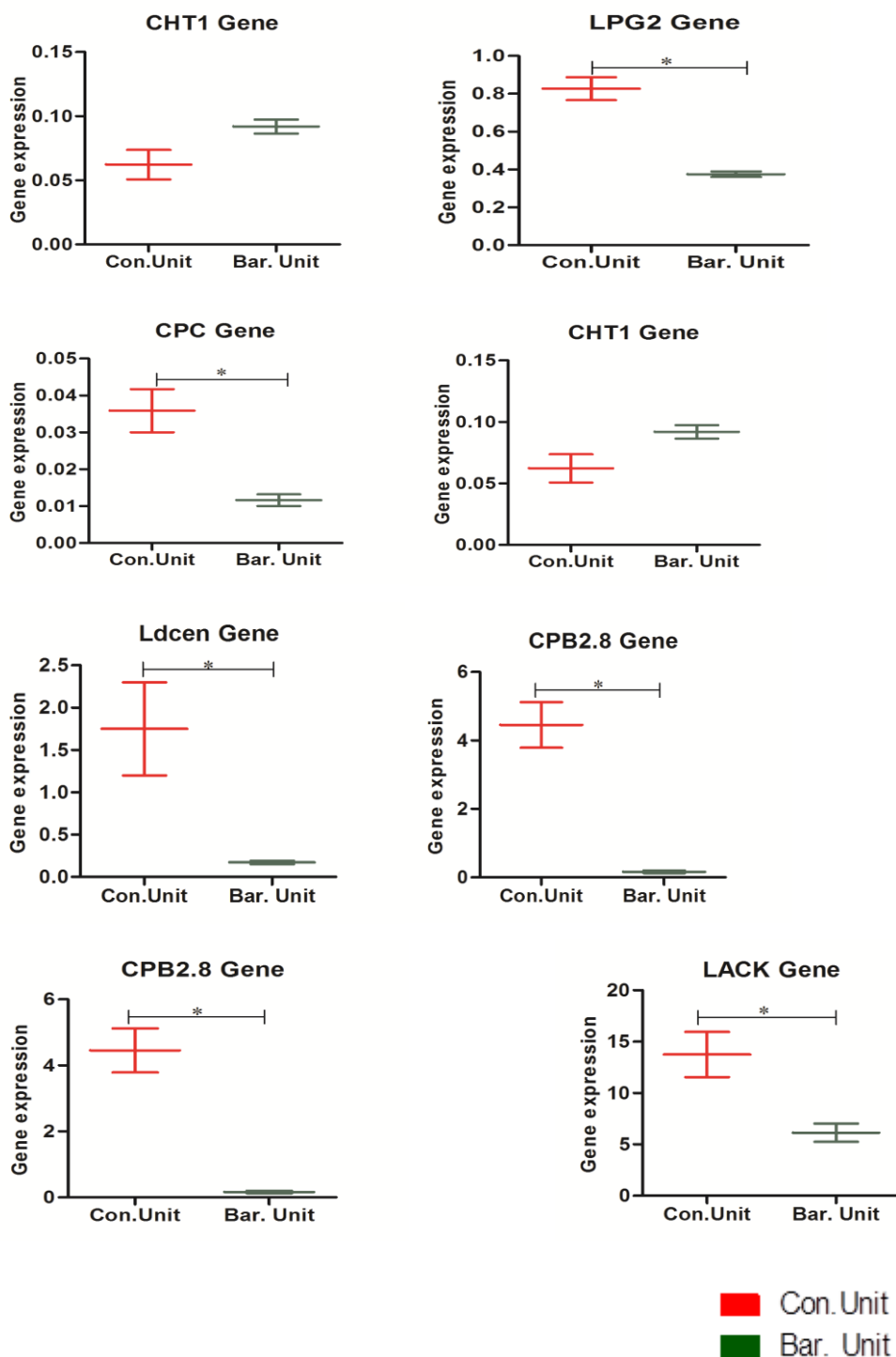
U937 control		Sample # 5	nm 1 abs. 27.41	A-260 27.41	ng/ul 1097
		A-280 13.13	260/280 2.09	260/230 1.81	
U937+Vir. L. mex		Sample # 5	nm 1 abs. 16.65	A-260 16.65	ng/ul 666.0
		A-280 8.013	260/280 2.08	260/230 2.11	
U937+Avir. L. mex		Sample # 5	nm 1 abs. 21.75	A-260 21.75	ng/ul 870.1
		A-280 10.42	260/280 2.09	260/230 2.13	
U937+Vir. L. SLA		Sample # 5	nm 1 abs. 22.40	A-260 22.40	ng/ul 895.8
		A-280 10.59	260/280 2.11	260/230 2.08	
U937+Avir. SLA		Sample # 5	nm 1 abs. 22.67	A-260 22.67	ng/ul 906.9
		A-280 10.91	260/280 2.08	260/230 1.71	
Mac6 control		Sample # 5	nm 1 abs. 32.19	A-260 32.19	ng/ul 1288
		A-280 15.57	260/280 2.07	260/230 2.12	
Mac6+Vir. L. mex		Sample # 5	nm 1 abs. 19.63	A-260 19.63	ng/ul 785.1
		A-280 9.358	260/280 2.10	260/230 2.20	
Mac6+Avir. L. mex		Sample # 4	nm 1 abs. 18.47	A-260 18.47	ng/ul 738.6
		A-280 8.908	260/280 2.07	260/230 2.11	
Mac6+Vir. SLA		Sample # 1	nm 1 abs. 32.68	A-260 32.68	ng/ul 1307
		A-280 15.44	260/280 2.12	260/230 1.60	
Mac6+Avir. SLA		Sample # 1	nm 1 abs. 35.11	A-260 35.11	ng/ul 1404
		A-280 16.82	260/280 2.09	260/230 1.78	
Macro control		Sample # 1	nm 1 abs. 19.97	A-260 19.97	ng/ul 798.6
		A-280 9.245	260/280 2.16	260/230 1.79	
Macro+Vir. L. mex		Sample # 1	nm 1 abs. 25.92	A-260 25.92	ng/ul 1037
		A-280 12.32	260/280 2.10	260/230 1.91	
Macro+Avir. L. mex		Sample # 1	nm 1 abs. 26.25	A-260 26.25	ng/ul 1050
		A-280 12.53	260/280 2.10	260/230 2.11	
Macro+Vir. L. SLA		Sample # 1	nm 1 abs. 35.16	A-260 35.16	ng/ul 1407
		A-280 16.93	260/280 2.08	260/230 1.51	
Macro+Avir. SLA		Sample # 1	nm 1 abs. 13.25	A-260 13.25	ng/ul 530.0
		A-280 6.459	260/280 2.05	260/230 1.49	

Appendix 10- Quantity analysis of RNA from human cell lines using the Nano-Drop spectrophotometer.

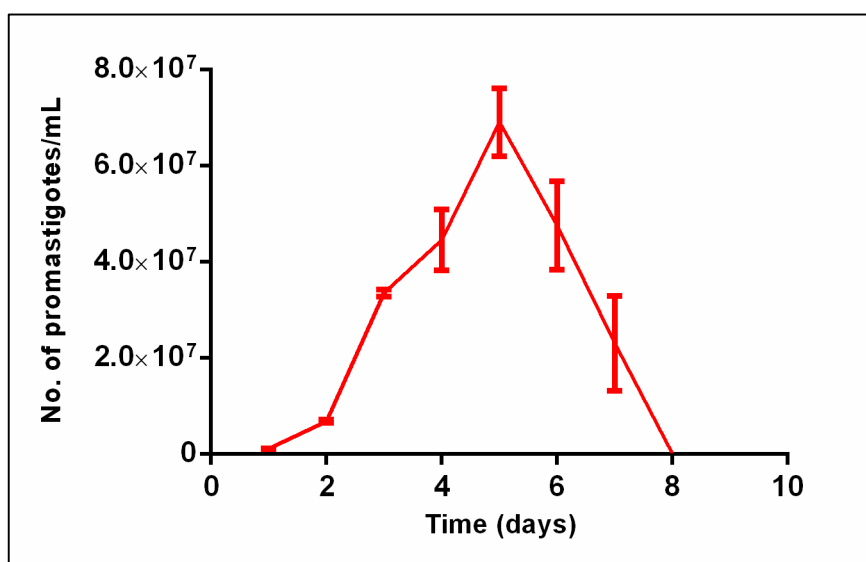
Appendix

Passage 1 mid log		Sample # <input type="text" value="2"/>	nm 1 abs. <input type="text" value="25.12"/>	A-260 <input type="text" value="25.12"/>	ng/ul <input type="text" value="1005"/>
		A-280 <input type="text" value="11.77"/>	260/280 <input type="text" value="2.13"/>	260/230 <input type="text" value="1.68"/>	
Passage 1 late log		Sample # <input type="text" value="2"/>	nm 1 abs. <input type="text" value="14.01"/>	A-260 <input type="text" value="14.01"/>	ng/ul <input type="text" value="560.4"/>
		A-280 <input type="text" value="6.537"/>	260/280 <input type="text" value="2.14"/>	260/230 <input type="text" value="2.14"/>	
Passage 20 mid log		Sample # <input type="text" value="2"/>	nm 1 abs. <input type="text" value="23.90"/>	A-260 <input type="text" value="23.90"/>	ng/ul <input type="text" value="956.1"/>
		A-280 <input type="text" value="11.01"/>	260/280 <input type="text" value="2.17"/>	260/230 <input type="text" value="1.76"/>	
Passage 20 late log		Sample # <input type="text" value="2"/>	nm 1 abs. <input type="text" value="13.17"/>	A-260 <input type="text" value="13.17"/>	ng/ul <input type="text" value="526.7"/>
		A-280 <input type="text" value="6.421"/>	260/280 <input type="text" value="2.05"/>	260/230 <input type="text" value="2.03"/>	
Passage 1		Sample # <input type="text" value="5"/>	nm 1 abs. <input type="text" value="32.19"/>	A-260 <input type="text" value="32.19"/>	ng/ul <input type="text" value="1288"/>
		A-280 <input type="text" value="15.57"/>	260/280 <input type="text" value="2.07"/>	260/230 <input type="text" value="2.12"/>	
Passage 7		Sample # <input type="text" value="5"/>	nm 1 abs. <input type="text" value="19.63"/>	A-260 <input type="text" value="19.63"/>	ng/ul <input type="text" value="785.1"/>
		A-280 <input type="text" value="9.358"/>	260/280 <input type="text" value="2.10"/>	260/230 <input type="text" value="2.20"/>	
Passage 20		Sample # <input type="text" value="4"/>	nm 1 abs. <input type="text" value="18.47"/>	A-260 <input type="text" value="18.47"/>	ng/ul <input type="text" value="738.6"/>
		A-280 <input type="text" value="8.908"/>	260/280 <input type="text" value="2.07"/>	260/230 <input type="text" value="2.11"/>	

Appendix 11- Quantity analysis of RNA from *L. mexicana* promastigotes using the Nano-Drop spectrophotometer

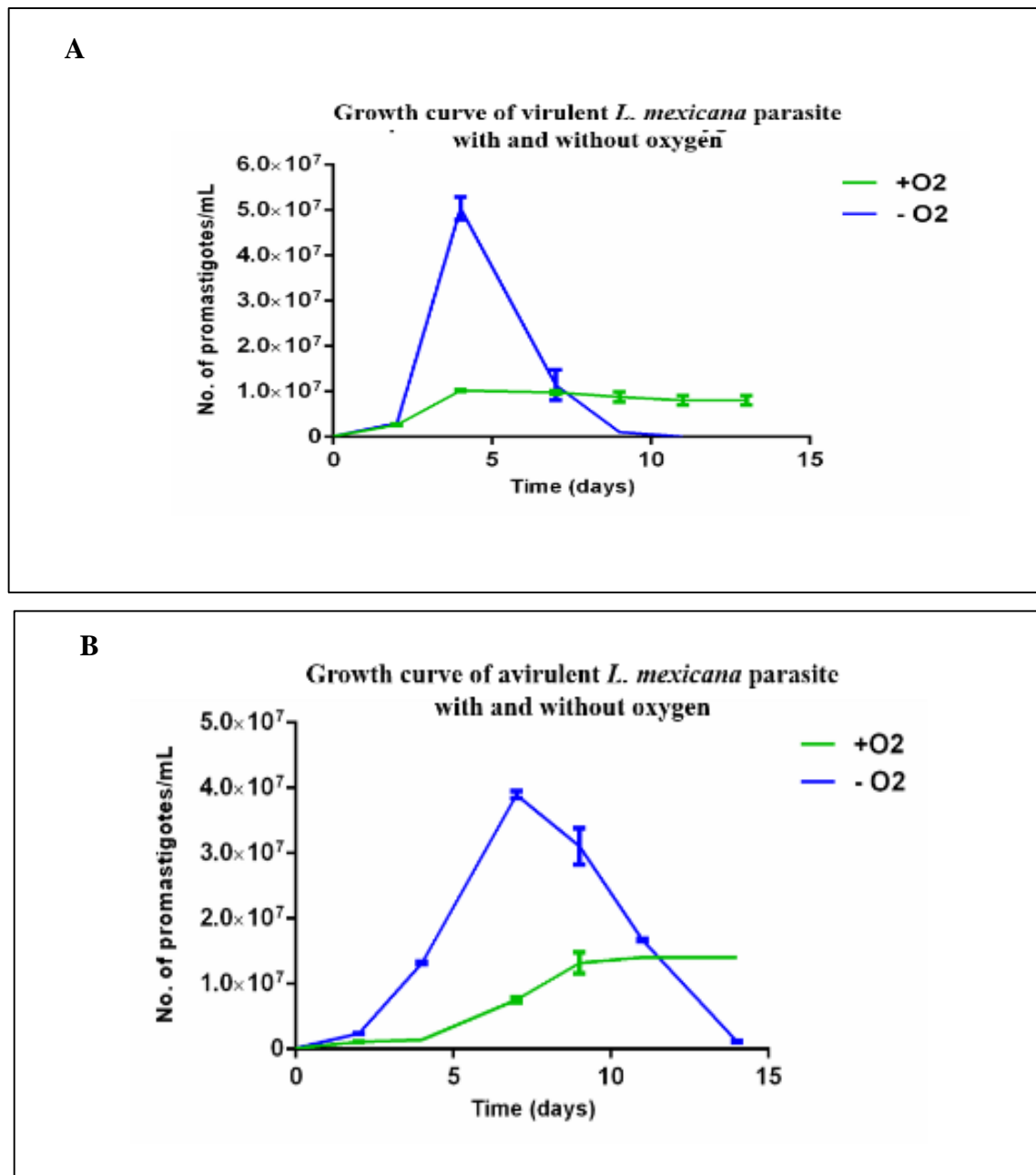


Appendix 12- The differences in associated gene expression profile in *L. mexicana* generated by passaging in Balb/c mice in Conventional unit and Barrier unit.
P<0.05 a using Mann–Whitney U test.



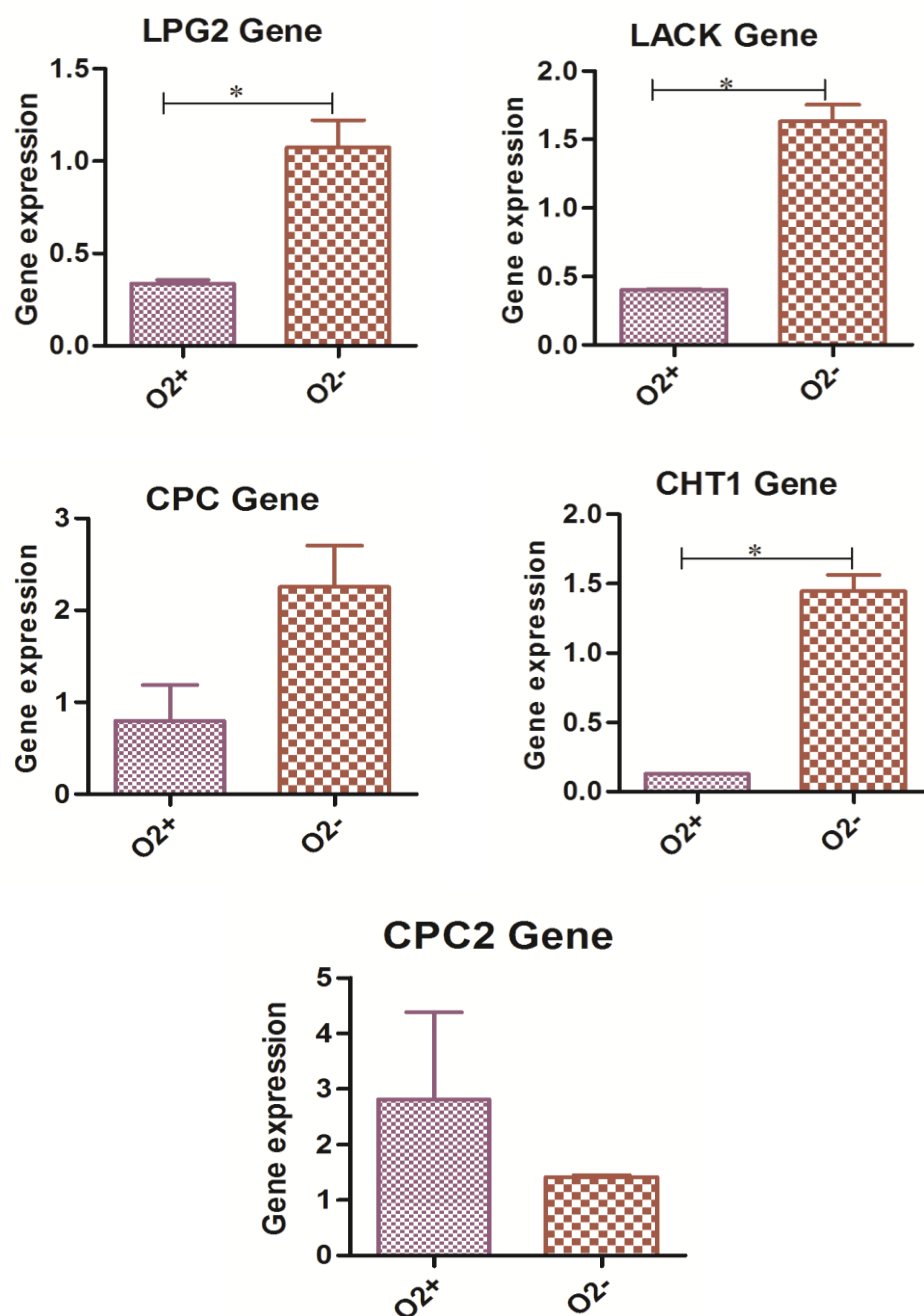
Appendix 13- *L. mexicana* promastigote growth curve span in high concentration

L. mexicana were cultured in Schneider media supplemented with 10% HFCS at 25°C. The graph was prepared by daily counting of parasite cultures which started at a concentration of 1×10^6 promastigotes per ml. The graph illustrate that the growth curve only last 8 days when the cultures were started with high parasite concentrations.



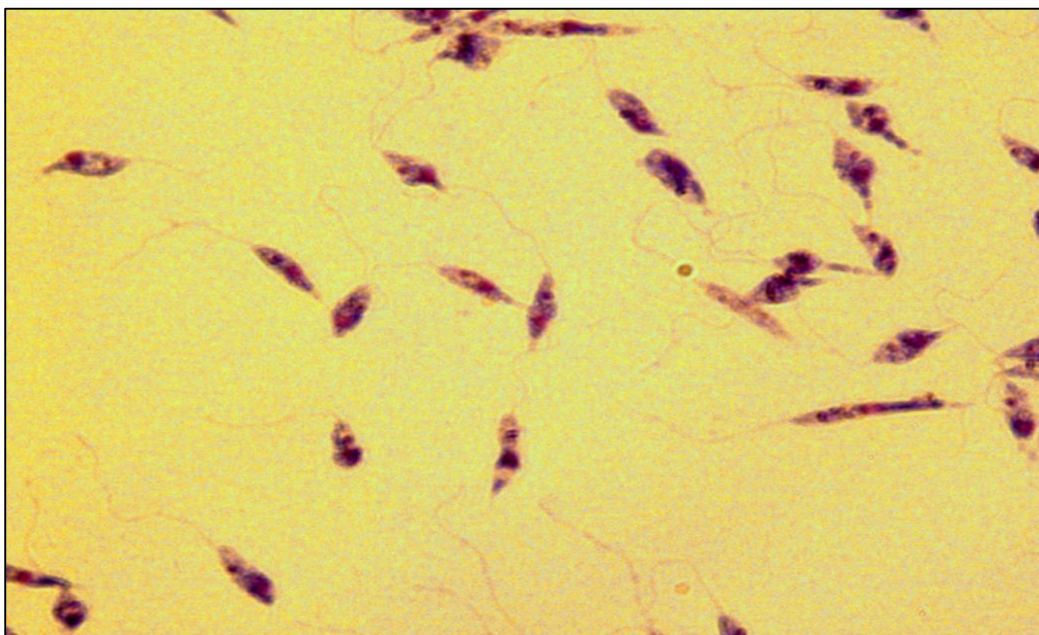
Appendix 14- Effect of oxygen on the parasites growth curves

Virulent and avirulent *L. mexicana* were cultured with and without oxygen at a concentration of 5×10^5 promastigotes per ml in Schneider media supplemented with 10% HFCS at 25°C. 10 μ l of each culture was fixed and counted daily using a Neubauer Hemocytometer. (A) virulent, (B) avirulent. The graphs represent the average of three independent experiments, which clearly shows that the stationary phase can be produced if the parasites growing in the presence of O₂. However, there is no stationary phase, when the parasites were growing without O₂. Each experiment consist of three cultures. Data are mean \pm SE.

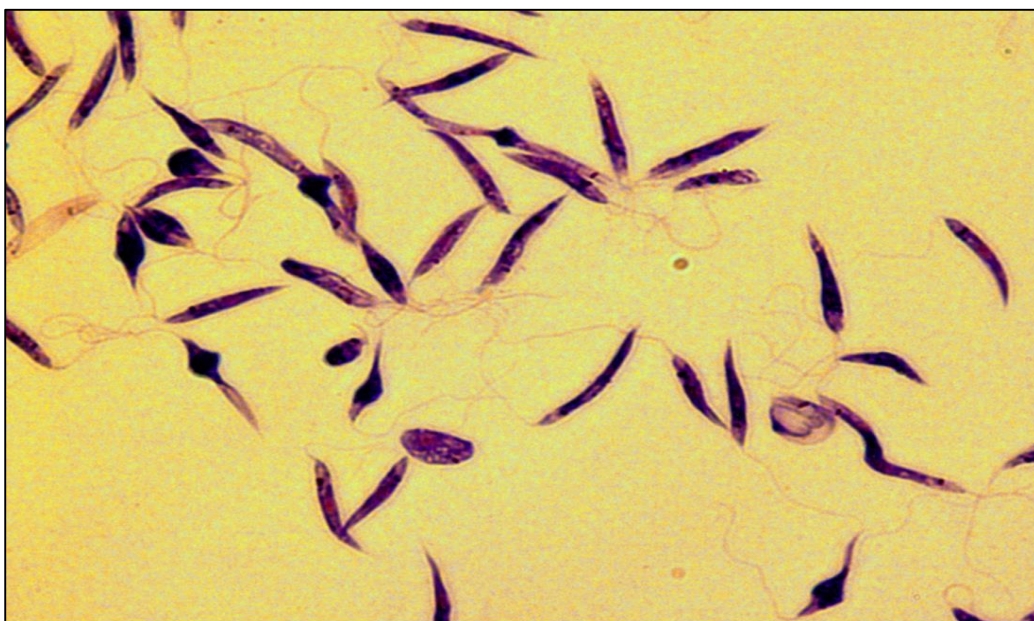


Appendix 15- Effect of oxygen on the expression of virulence associated genes

RNA was extracted from the late log stage of virulent *L. mexicana* promastigotes growing in the presence and absence of oxygen. RNA was converted into cDNA which was used as a DNA template for qPCR analysis. The results showed target genes were expressed more when the parasites were growing without oxygen. $P < 0.05$ using Mann-Whitney U test



Virulent L. mexicana (P1)



Avirulent L. mexicana (P20)

Appendix 16- virulent and avirulent *L. mexicana* promastigotes